Passive and Acquired Immunity to Respiratory Syncytial Virus in Young Children in Rural Kenya

Thesis

PASSIVE AND ACQUIRED IMMUNITY TO RESPIRATORY SYNCYTIAL VIRUS IN YOUNG CHILDREN IN RURAL KENYA

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DEDICATION

To the memory of my late grandfather, JH Odonde, and for my parents, Mama and Dad, who always instilled the value of hard work.

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Declaration

I, **Beldinah Rachel Ochola-Opiyo**, certify that the work embodied in this thesis is the result of my own investigations except where reference has been made to the published literature.

I declare that this work has not already been accepted in substance, or is being currently submitted in candidature for any other degree.

Candidate: Beldinah R. Ochola-Opiyo

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Signature:...

Date: 17-12-2007

Director of studies: Dr. D. James Nokes

Signature:.

Date: 18-6-08

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ABBREVIATIONS

Ab	*Antibody*
ARI	*Acute respiratory infection*
AU	*Arbitrary units*
BPD	*Bronchopulmonary dysplasia*
CF	*Complement fixation assay*
CHD	*Congenital heart disease*
CI	*Confidence intervals*
CTL	*Cytotoxic T-lymphocytes*
CYO	*Child years observation*
DC	*Dendritic cells*
DFA	*Direct fluorescence assay*
ELISA	*Enzyme-linked immunosorbent assay*
ELISA-CSR	*Enzyme-linked immunosorbent assay-confirmed serological response*
Epi-DSS	*Epidemiological-demographic surveillance system*
F protein	*Fusion protein*
FDA	*Food and Drug Administration, USA*
G protein	*Glycoprotein*
GA	*Gestational age*
HIV	*Human immunodeficiency virus*
IFAT	*Indirect immunofluorescence antigen test*
IFAT-CSR	*Indirect immunofluorescence antigen test-confirmed serological response*
IgA	*Immunoglobulin A*
IgE	*Immunoglobulin E*
IgG	*Immunoglobulin G*
IgM	*Immunoglobulin M*
KDH	*Kilifi District Hospital*
KM	*Kaplan Meier*
Log AU	*Logarithm arbitrary units*
LRTD	*Lower respiratory tract disease*
LRTI	*Lower respiratory tract infection*
mac	*Macrophages*
matAb	*Maternal antibody*
MBL	*Mannose binding lectin*
mcAb	*Monoclonal antibody*
MCHC	*Maternal child health clinic*
MFAT	*Membrane fluorescence antibody technique*
MHC	*Major histocompatibility complex*
min	*Minutes*
MIP	*Macrophage inflammatory protein*
MNA	*Microneutralization assay*
mPCR	*Multiplex polymerase chain reaction*
NA	*Neutralization assay*
NPS	*Nasal pharyngeal secretion*
NW	*Nasal wash*
NWB	*Nasal wash bulb*

OD	*Optical density*
OP	*Out-patient*
RANTES	*Regulated on activation, normal T-cells expressed and secreted*
RFLP	*Restriction fragment length polymorphism*
RNA	*Ribomucleic acid*
RSV	*Respiratory syncytial virus*
RT-PCR	*Reverse transcriptase polymerase chain reaction*
s	*Seconds*
sd	*Standard deviation*
$T_{1/2}$	*Half life*
T_H	*T- helper cell*
TLR	*Toll-like receptor*
TNF	*Tumour necrosis factor*
URT	*Upper respiratory tract*
URTI	*Upper respiratory tract infection*
WB	*Western blot*
wfh	*Weight-for-height*
WHO	*World Health Organization*

ABSTRACT

The rate of decay and the subsequent antibody responses to RSV are poorly defined in young infants and children who possess maternally derived respiratory syncytial virus (RSV) antibodies. A birth cohort from rural Kenya was studied intensively to monitor infections from whom blood samples were collected at regular intervals to describe the age-related serological characteristics. A simple linear regression model was used to calculate the rate of RSV-specific maternal antibody decline. In addition, the effect of risk factors on cord blood titres was investigated. Using the random effects model, the half-life of RSV maternal antibodies was calculated to be 79 days. Although 97% of infants were born with RSV-specific maternal antibodies, it was noted that infants who went on to experience an infection in early life, had lower starting titres of RSV maternal antibodies in comparison to infants who did not have any clinically confirmed infection in the first 6 months. Additionally, clinically confirmed infections within the first 6 months of life had no effect on the rate of decay of maternal antibodies.

Both RSV group A and B were seen to circulate in the community in varying amounts, with RSV A seen to be the most dominant strain in the 4 epidemics experienced by the cohort. The same RSV strain was observed to cause both RSV-associated upper respiratory tract and lower respiratory tract infections. It was observed that there existed patterns of antibody decay and acquisition of RSV-specific immune responses that groups of children appeared to follow. A group of infants were seen to undergo clinical infection that was subsequently confirmed by the ELISA, or were observed to seroconvert in the absence of clinical symptoms, or infants did not experience any infection at all despite experiencing at least 2 epidemics. Post-infection dynamics showed classical boosting of RSV antibodies, which either quickly waned or remained elevated over time. Furthermore, the risk of re-infection with clinically identified RSV illness decreased with age, from an initial infection rate of 252 to 41/1000 child years observation.

In conclusion, the evidence shows that RSV maternal antibodies provide some protection against severe disease during the first 6-7 months. Since there is efficient transfer of antibodies from mother to child, maternal vaccination against RSV may be a useful strategy to consider. However, our observations show that these antibodies decline quickly, hence childhood vaccines should also be taken into consideration to augment the immune responses.

CHAPTER ONE

Introduction

1.1 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is the single most important viral cause of lower respiratory tract infection (LRTI) during infancy and early childhood worldwide (Chanock & Parrott, 1965, Loscertales et al., 2002, Roca et al., 2002, Zambon et al., 2001). Evidence of RSV infection has been found in all investigated geographic areas, and it has shown similar characteristics in regions with different climates (Cane et al., 1999, Coggins et al., 1998, Peret et al., 1998). Outbreaks appear as predictable annual events, during the cold season in temperate climates and usually during the rainy season in tropical ones (Cane, 2001, Hall et al., 1990). Peak occurrences of morbidity and mortality have been seen to occur in infants younger than 3 months of age (Welliver, 1988).

In developing countries, few studies have attempted to quantify the importance of acute lower respiratory infections (ALRI) such as bronchiolitis and pneumonia caused by RSV (Robertson et al., 2004). Additionally, risk factors have only been poorly defined (Simoes, 1999) despite the fact that LRTIs are among one of the leading causes of infant and childhood deaths in Africa (Bryce et al., 2005, Loscertales et al., 2002, Williams et al., 2002). It is now well established that numerous components of the immune system are activated during RSV infection (Brandenburg et al., 2000, Cherrie et al., 1992, Hall et al., 1991, Hornsleth et al., 2001, Murphy et al., 1986) although the critical components contributing to diverse RSV-associated disease outcomes still remain poorly defined. The role of RSV antibodies (Abs) in infant protection in the developing world where the epidemiology of RSV and related infections may differ from that in developed regions, is only now being evaluated (Roca et al.,

2002, Roca et al., 2003). Furthermore, there is still a paucity of information on the efficiency of transfer of RSV-specific maternal antibodies (matAbs) to the child, if these matAbs protect younger infants against early onset of infection and hence RSV disease, and if the latter are strain-specific. It is also not fully known to what extent the impact of antigenic diversity has on RSV epidemiology (Cane, 2001). Conflicting data on the nature of immunity in infancy and the development of acquired immunity still remains. Furthermore, very little data of this nature exists from the developing world.

This research project aimed to shed light on some of these issues, advancing our understanding on the requirements for vaccines in early infancy and/or mothers, and by providing baseline data for future immunization programmes. This was achieved by obtaining information on the distribution of circulating RSV genotypes in the community, the levels of RSV-specific matAbs and their decay rate. Furthermore, the putative threshold level of Abs for protection was calculated. The project was undertaken within the existing framework of a recently completed longitudinal birth cohort study in Kilifi District, Kenya, in which a birth cohort of approximately 600 children was recruited in a staggered fashion in early 2002 and 2003, respectively. These were monitored for respiratory infection by active home visits and passive referrals to the research clinic at Kilifi District Hospital (KDH) (Nokes et al., 2004; 2007).

1.2 Objectives

The main objective of this study was to elucidate the role of passive and active immunity to RSV infection in infants and young children in a developing country setting, specifically in Kenyan children. The specific objectives included:

- The description of the decay function for passive anti-RSV IgG in children less than one year of age, and its association with child risk factors such as prematurity, birth weight, malnutrition, malaria, or other environmental measures (*e.g.* elder siblings, pollution).
- The quantification of the relationship between maternally derived RSV Ab and protection against infection, or disease type (upper respiratory tract infection (URTI) or LRTI), and determination of thresholds of passive immunity against these outcomes.
- The characterization by molecular methods of the genetic diversity (group and genotype) of RSV infecting infants in the study population.
- The investigation of RSV specific Ab responsiveness and kinetics following primary infection as well as re-infections, in relation to age at infection, infecting strain, homologous or heterologous matAb, and disease severity.

1.3 The Scope of the Thesis

Following this introduction, an overview is presented on the burden of RSV disease as an indication of its importance as a childhood disease both in the developed and developing country settings. A concise review follows in chapter two of the biology of RSV, its discovery, its structure, life cycle, and molecular epidemiology through to the current status of vaccine availability. Chapter three describes the role of matAbs in protection against severe RSV-associated disease in early childhood, specifically in a developing country setting drawing from earlier studies carried out in the developed world. Chapter four reviews the methodologies used in various studies, and based on the analysis, selected the enzyme-linked immunosorbent assay (ELISA). Chapter five presents results from using the ELISA methodology to investigate the characteristics of passive and acquired immune responses in

early childhood. Chapter six examines the circulating genotypes in Kilifi District during the four years of follow up. Chapter seven deals with the characterization of the decay of RSV-specific matAbs. Chapter eight examines the relationship between RSV infection and pre-existing matAb levels. In addition, the immune responses to RSV post-matAb were further explored. The final chapter, nine, summarizes the findings of the work presented and outlines the implications for vaccine design. The chapter also identifies limitation of the research and provides suggested areas for further research.

1.4 The Burden of RSV Disease

Acute respiratory diseases continue to be important causes of childhood morbidity and mortality worldwide. Whereas URTIs are very common, and rarely life-threatening, LRTIs are responsible for more severe illnesses such as pneumonia and bronchiolitis. They contribute to approximately 4 million deaths each year (Rudan et al., 2004). Figure 1.1 illustrates that pneumonia accounts for one in five child deaths.

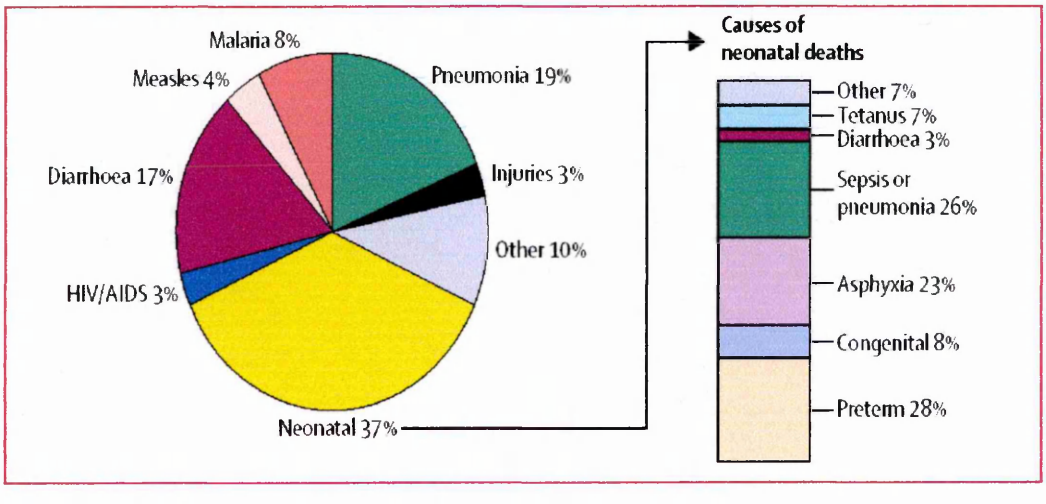


Figure 1.1. Major causes of death in children younger than age 5 years and in neonates (yearly average for 2000-03) (Bryce et al., 2005)

Forty-six percent of deaths as a result of pneumonia in children under 5 years of age worldwide, were noted to occur in the WHO Africa region (6 WHO regions have been defined and include Africa, Americas, Eastern Mediterranean, Europe, Southeast Asia and Western Pacific), with an additional 29% occurring in the Southeast Asia region (Bryce et al., 2005). From our observations at the KDH in 2004, out of a total of 5019 admissions to the paediatric wards, LRTIs final diagnosis constituted 26% of the total, and LRTI-associated deaths were estimated at 15% as given in Figure 1.2 (Kilifi epidemiological-demographic surveillance system; Epi-DSS). The observed deaths by cause in this region were consistent with worldwide estimates.

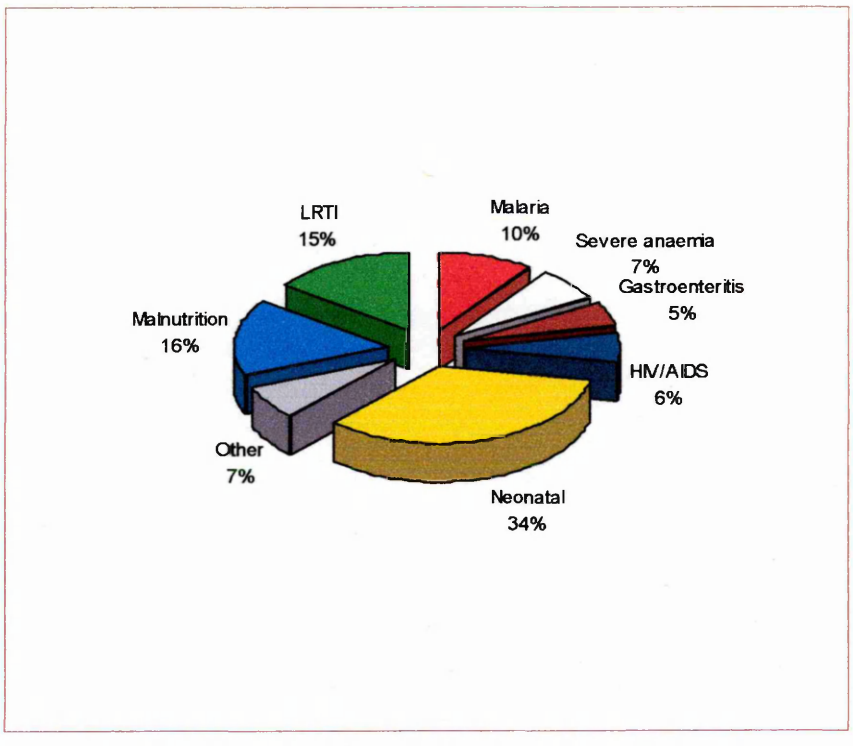


Figure 1.2. Number of deaths in children admitted to the KDH, Kilifi Kenya (yearly average for 2004).

Efforts in prevention and control of childhood deaths by major causes of mortality and morbidity including pneumonia were recently renewed worldwide (Bryce et al., 2005). There is rapidly accumulating evidence for RSV being a significant pathogen in young children as well as a major contributor to lower respiratory tract disease (LRTD) in infants as similarly described in developed countries (Robertson et al., 2004). RSV has been found to be pathogenic in young infants throughout the world (Bedoya et al., 1996, Cox et al., 1998, Hacimustafaoglu et al., 2004, Hacking & Hull, 2002, Madhi et al., 2000, Nokes et al., 2004, Roca et al., 2001, Scott et al., 2004, Venter et al., 2001, Weber et al., 1998a). Additionally, it was observed that although bacterial conjugate vaccines prevented between 20-37% of radiologically confirmed pneumonia, the overall reduction in the burden of clinically diagnosed LRTI was only 6-9% (Cutts et al., 2005, Mulholland et al., 1997), RSV remained a major cause of LRTI-associated morbidity.

The two hallmarks of this viral infection in the first several years of life have been described as the notably early infection attended by serious disease, especially under 6 months of age, and repeated infection with eventual development of immunity to serious lower respiratory RSV-associated disease (Fernald et al., 1983, Henderson et al., 1979, Parrott et al., 1973). In this respect therefore, RSV remains unique among the more than 200 serologically distinct viruses that have been implicated in the aetiology of respiratory tract infection (Minnich & Ray, 1980, Olszewska et al., 2002), in that the incidence of serious disease is highest among 2-month-old children, who possess matAbs (Boeck, 1996, Lamprecht et al., 1976).

By 2 years of age, virtually all children have been infected with the virus (Glezen et al., 1986). A high incidence of hospitalization associated with RSV infection has also been described (Glezen, 2001, Shay et al., 1999). From a longitudinal study spanning 20 years (1973-1993),

in which babies were recruited from birth (full-term) hospitalization rates were estimated to be 23.8% of all RSV culture-positive patients (Fisher et al., 1997). Another similar follow up study that investigated American children and which spanned a 17 year period (1980-1996), was carried out through the US National Hospital Discharge Survey. This study estimated 1.65 million hospitalizations for bronchiolitis occurred in the under 5 year age group, whilst the under 6 month and under 1 year old age bracket accounted for nearly 57 and 81% respectively (Shay et al., 1999). This translated to an incidence of 3-9 per 1000 children younger than 1 year being hospitalized for this condition. Children with underlying conditions such as bronchopulmonary dysplasia (BPD), congenital heart disease (CHD), or born at ≤ 28 weeks' gestation, were observed to have an increased risk of RSV hospitalization until 24 months of age (Boyce et al., 2000). Therefore, WHO designated RSV a major target of research and therapy (www.who.int/vaccine_research/diseases/portfolio/en/index.html).

Reports have further noted that within developing countries, the highest incidence of RSV-LRTI occurs in infants < 6 months of age, with two-thirds or RSV-LRTIs occurring in children younger than 2 years of age (Loscertales et al., 2002). Moreover, the burden and severity of disease was high, with the incidence of RSV-associated LRTI being reported as 2.4-4.8 times the rate of that seen in the US (Wright & Cutts, 2000). Incidence rates of RSV however varied from between 18 per 1000 children younger than one year hospitalized with RSV-associated disease as in southern Israel (Dagan et al., 1993) to 198 per 1000 children years for children up to 18 months in a community based study from Colombia (Borrero et al., 1990). This compared with admission data from KDH during 2002-2005, in which the incidence of RSV at admission was 60 per 1000 child year observations/annum (Figure 1.3).

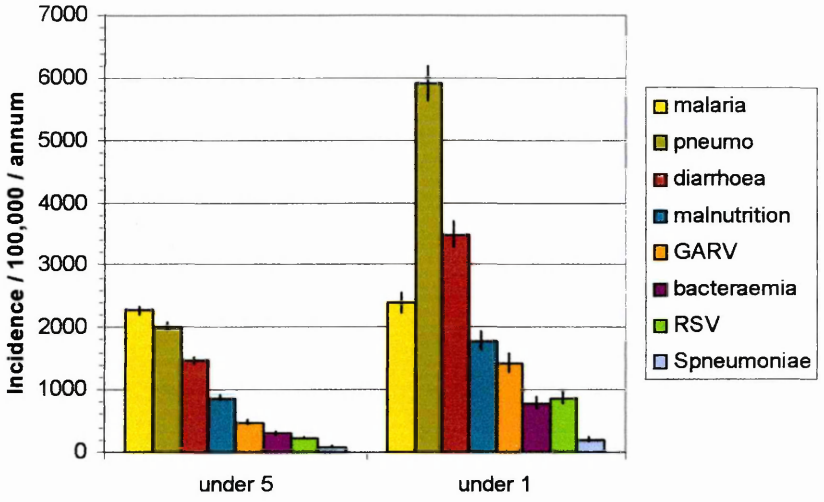


Figure 1.3. Comparative incidence of admissions from 2002-2005 of various childhood diseases at KDH (Kilifi Epi-DSS) stratified into infants and children under 5 years. Also depicted is the contribution of RSV and *S. pneumoniae* to respiratory illnesses. Pneumo stands for severe and very severe pneumonia (WHO defined); GARV for group A rotavirus.

The gap in knowledge with respect to the role of Abs in RSV immunity and pathogenesis is yet to be fully elucidated in the different age groups and especially due to matAbs. There also remains a significant deficit in our understanding with regards to the most appropriate correlates of protection and the ideal or desirable immune response to RSV infection as noted following the harmful immune response elicited by the formalin inactivated RSV vaccine (refer further to section 2.8). This uncertainty has hampered vaccine intervention. These have been especially aimed to reduce the burden of RSV disease. Despite this, there have been renewed efforts focussing on these areas. Significant advances leading to some understanding of the nature of RSV immunity age-related difference in Ab responses to RSV infection during early life, the extent of genotype variation *e.g.* it has been shown that from one epidemic to the next, different genotypes contribute to the makeup of the presently circulating strain at the local level (chapter 6) and the pathogenesis of RSV infection since its discovery have been realized.

This present study aims to examine further the protective role of matAb against RSV disease, its duration and factors affecting the latter by evaluating regularly collected serum samples from infants recuited from birth in the coastal community of Kilifi, Kenya.

CHAPTER TWO

The Biology of Respiratory Syncytial Virus

2.1 The Pathogen- Historical Overview

In October 1955, at the Forest Glen facility of the Walter Reed Army Institute of Research in Washington, D.C., an apparent epizootic of sneezing, coughing and mucopurulent nasal discharge was observed in a group of young chimpanzees housed there (Morris et al., 1956). Investigators went on to culture the nasal secretions obtained from these symptomatic chimps in human liver epithelial cells (Chang) and recovered an unrecognized virus which they subsequently named the chimpanzee coryza agent (CCA). They went on to show that it was causally related to the monkeys' URTI. Seed virus prepared from the original CCA isolation was also shown to induce infection and similar respiratory symptoms in other young chimps, but failed to induce detectable infections in guinea pigs, mice, rats or chicken embryos. It was further noted that there was unintentional spread of CCA between infected and uninfected chimps housed in a common facility. Additionally, a laboratory worker seemed to become infected with CCA following contact with the infected chimps.

Soon after this discovery and subsequent recovery of CCA, Chanock and colleagues (Chanock et al., 1957, Chanock & Finberg, 1957) went on to isolate a CCA-like virus from an infant with bronchopneumonia (Long agent) and another from an infant with bronchiolitis (Snyder). These agents were shown to be serologically and phenotypically similar to CCA, as characterized by the viruses' cytopathic effect on cells in culture as well as by their neutralization by anti-CCA animal sera. Of note, was their ability to induce syncytia and multinucleated giant cells in Chang cells. Therefore, respiratory syncytial virus (RSV) was suggested as a more suitable name than CCA, due to its propensity to induce syncytia

formation in tissue culture. Subsequently, RSV was recovered from 57% of young infants with bronchiolitis or pneumonia during a 5-month study period (Chanock et al., 1957, Chanock & Finberg, 1957). RSV was also recovered from older children with bronchopneumonia and bronchiolitis.

2.2 Structure and Genome

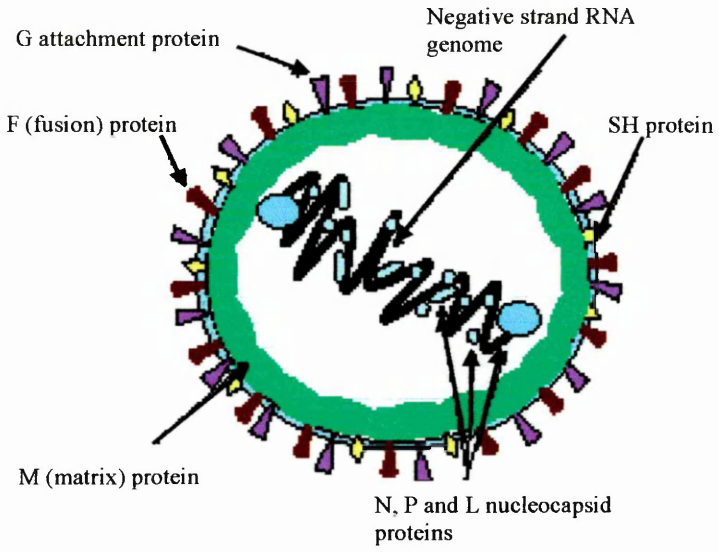


Figure 2.1. Structure of respiratory syncytial virus (Hacking & Hull, 2002)

RSV is classified in the genus *Pneumovirus* of the family *Paramyxoviridae*. The RSV virion is about 200 nm in size and consists of a nucleocapsid with a lipid envelope (Figure 2.1). The nucleocapsid has been shown to be a symmetrical helix with a diameter of 12-15 nm. The RSV genome is composed of a single stranded negative sense, non-segmented RNA and comprises 15,200 nucleotides encoding 11 viral proteins (Figure 2.2 and Table 2.1). The lipid bilayer is derived from the host plasma membrane and contains virally encoded transmembrane surface glycoproteins, which are 11-20 nm in size and closely spaced at intervals of 6-10 nm. There are 3 transmembrane glycoproteins, *i.e.* the attachment glycoprotein (G), the fusion protein (F) and the small hydrophobic protein (SH). As well, two matrix proteins, M and M2 (or 22K); three proteins associated with the nucleocapsid, nucleocapsid (N), phosphoprotein (P) and polymerase (L); and two non-structural proteins, NS1 and NS2 have been described.

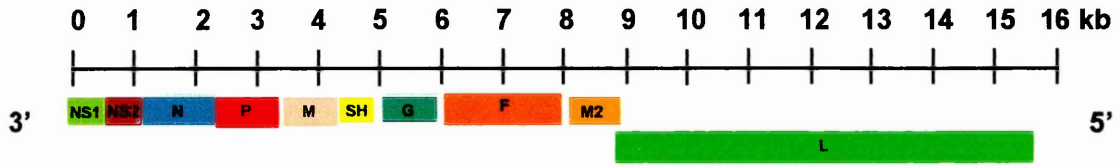


Figure 2.2. Schematic representation of the respiratory syncytial virus (hRSV) ssRNA (negative-strand) genome based on GenBank accession no. NC_001781. (kb denotes kilobase)

Table 2.1. RSV gene and protein lengths. (Collins et al., 1996, Collins et al., 1990, Collins & Wertz, 1985, Hacking & Hull, 2002, Olmsted & Collins, 1989, Pastey & Samal, 1995)

Gene	Gene Length[a]	Protein Length[b]	Functions	Comments
Nucleocapsid-associated proteins[c]				
N	1 203	391	Nucleocapsid protein: nucleoprotein essential for transcriptional activity	Binds tightly to genomic and R1 RNAs
P	914	241	Nucleocapsid protein: phosphoprotein essential for transcriptional activity	Exhibits anomalous electrophoretic mobility due to charged residues
L	6 578	2 165	Nucleocapsid protein: RNA polymerase	Contains highly conserved putative functional domains
Transmembrane surface proteins[c]				
F	1 903	574	Fusion protein: viral entry and syncytia formation	Major protective antigen
G	923	298	Glycoprotein: viral attachment to the cell	Highly divergent between RSV antigenic subgroups; major protective antigen
SH (1A)	410	64	Small hydrophobic protein: function unknown	
Matrix proteins[c]				
M	958	256	Matrix protein: viral assembly	Counterpart to the typical paramyxovirus M protein
M2 (or 22K)	961	194	M2-1:transcription elongation factor M2-2: regulation of viral transcription	Unique to pneumoviruses; M2-1 and M2-2 overlapping genes encoded by the 1st and 2nd open reading frame of M2 gene respectively
Nonstructural proteins				
NS1 (1C)	532	139	Non-structural proteins: anti-interferon α and β activity	Unique to pneumoviruses
NS2 (1B)	503	124		Unique to pneumoviruses

[a]gene length in nucleotides. The encoded mRNA would be this exact length plus a 3' poly (A) tail.
[b]Length in amino acids of complete, unprocessed protein deduced from gene sequence
[c]Virion structural proteins

The F and G proteins have been shown to be important antigenically as a result of their ability to stimulate the production of neutralizing Ab responses (Anderson et al., 1988, Olmsted et al., 1986, Taylor et al., 1984, Walsh et al., 1984). Their importance is emphasized by the observation that effective humoral immunity is only conferred by Abs to these proteins as reviewed by Hacking and Hull (2002).

2.3 The Replication Cycle of RSV

RSV predominantly infects the airway epithelial cells (nose, large and small airways). For infection to occur, the first step is viral attachment to the cell through the G glycoprotein (Levine et al., 1987, Martinez & Melero, 2000, Schlender et al., 2003, Smith et al., 2002) followed by fusion of the viral envelope or infected cell membranes with uninfected cell membranes via the F protein (Smith et al., 2002, Walsh & Hruska, 1983). The viral envelope is then incorporated into the cell membrane and the nucleocapsid released into the cytoplasm. RNA replication and transcription occurs followed by viral assembly through a series of interactions mediated by the M protein which in turn also co-ordinates assembly of the envelope proteins (F and G) with nucleocapsid proteins into new virions (reviewed by Hacking & Hull, 2002). The packaged viral particles leave the cell either through fusion with adjacent cells or following cell rupture (Figure 2.3).

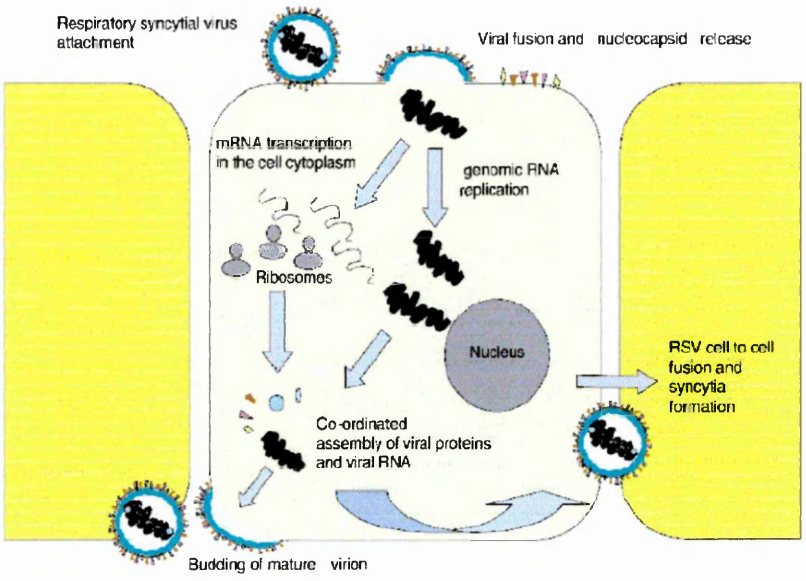


Figure 2.3. A schematic representation of the RSV replication cycle. RSV attachment occurs via the G protein. Budding appears to be the reverse of penetration and occurs *in vitro* on the apical cell surface. (Hacking & Hull, 2002).

2.4 Molecular Epidemiology

2.4.1 Group variability

Two antigenic groups, A and B, have been described based originally on serological reactivity both with monoclonal Abs (Anderson et al., 1985, Gimenez et al., 1984, Gimenez et al., 1986, Mufson et al., 1985) and polyclonal animal sera (Coates et al., 1963). These were later shown to be distinct at the nucleotide level. The nucleotide sequence similarity between groups ranges from 53% for G protein to 86% for N protein (Johnson & Collins, 1989, Johnson et al., 1987b). Additionally, there exist differences in the apparent size of the F1 and F2 cleavage products, and the P proteins between the 2 groups as detected by SDS-PAGE (Gimenez et al., 1986, Norrby et al., 1986). However, the most variable protein between these groups is the G protein and up to 47% amino acid variability between prototype strains of the different groups was earlier demonstrated (Johnson et al., 1987a).

2.4.2 Strain variability

In addition to group variability, it was noted that numerous strains existed in both groups. The latter showed variability amongst themselves and between themselves, *i.e.* intra- and inter-group antigenic differences. Within groups, differences between RSV was initially demonstrated using monoclonal Abs (Anderson et al., 1991, Orvell et al., 1987, Storch & Park, 1987). These results were soon confirmed by ribonuclease A (RNase A) mismatch cleavage method (Cristina et al., 1991, Storch et al., 1991) and nucleotide sequencing of the G gene where up to 20% amino acid variability was noted within the group A (Cane et al., 1991) and 9% in group B (Sullender et al., 1991).

Infection and re-infection of infants with RSV occurs despite the existence of serum anti-viral Abs, which have been noted to provide protection in animal models (Walsh et al., 1984). Antigenic variation may play an important role in aiding immune evasion.

The 2 transmembrane surface glycoproteins, G and F, appear to be the most important of the RSV proteins in inducing protective immunity (Connors et al., 1991, Glezen et al., 1986). Antigenic variation in RSV groups and within groups resides mainly in the C-terminal third of the G glycoprotein and to a lesser extent the F glycoproteins. Moreover in G proteins, mutations (insertions, deletions, replacement or substitutions) can lead to the progressive accumulation of amino acid changes within the variable regions (Cane & Pringle, 1995, Frabasile et al., 2003, Martinez et al., 1999, Sullender et al., 1991, Trento et al., 2003), with several studies highlighting a high number of nucleotide substitutions causing amino acid changes in both RSV groups (Coggins et al., 1998, Martinez et al., 1999, Sullender et al., 1991, Venter et al., 2001). The high ratio of non-synonymous mutations to synonymous

mutations implies a selective pressure on viruses of both groups. The regions of amino acids in which these changes are found are on either side of a putative binding site (Figure 2.4).

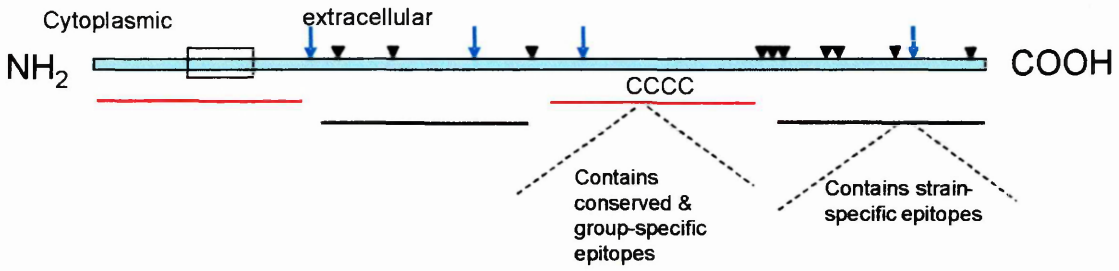


Figure 2.4: Schema of primary structure of G protein. Indicated are the cytoplasmic, transmembrane (▭) and extracellular domains; cysteine residues (CCCC) of the putative receptor binding site, potential sites for N- (▼), and O-glycosylation (▼), the presence and/or position of these sites varies between isolates. Also indicated is the conserved (—) and variable (—) segments of the protein. Adapted from (Cane, 1993, Melero et al., 1997, Palomo et al., 2000).

Mutation rates for the G glycoprotein of RSV have been said to be similar to other quickly evolving respiratory virus surface proteins, such as hemagglutinin protein of influenza A, being reported to be 1.83×10^{-3} and 1.95×10^{-3} nucleotide substitutions/site/year for RSV A and B, respectively (Zlateva et al., 2005, Zlateva et al., 2004). However, it has been proposed that natural RSV B isolates accommodate more drastic changes in their attachment G proteins (Zlateva et al., 2005).

It has been shown that during primary infections, the carboxy terminal region of the G protein is recognized by human Abs (Cane et al., 1996, Jones et al., 2002, Palomo et al., 2000). Moreover, infants exhibit responses to the G protein that are specific to the infecting genotype (Cane et al., 1996, McGill et al., 2004). In addition to these responses being dependent on the infecting genotype, recognition was shown to be also dependent to a great degree on the amino acid sequences of the peptides used as targets in the assay. There appears to be more than one antigenic site or several overlapping epitopes on RSV as has been previously described for

influenza virus haemagglutinin (Schoofs et al., 1988). However, these Ab responses have not been analyzed with respect to their ability to neutralize the virus.

The response to these proteins is complex due to the diversity in the types of neutralization responses. From studies carried out to characterize the *in vitro* function of specific epitopes through their interaction to their respective monoclonal antibodies (McAbs), neutralization or enhancement by these virus-specific Abs (Anderson et al., 1988, Gimenez et al., 1996) were described. RSV-specific McAbs were thus classified into three groups: (i) McAbs possessing neutralizing activity, (ii) McAbs possessing only enhancing activity, and (iii) McAbs possessing both neutralizing and enhancing activities. Within this framework, neutralization responses have been described as being partial (strain-specific) or complete (cross-protective) (Anderson et al., 1988). It is thus possible that following infection, varying amounts of each category may be induced on an individual level. It is proposed that a balance of both activities determines the net activities of sera specific to RSV (Gimenez et al., 1996). If for some reason or another, an imbalance in the Ab categories occur, this could result in suppression of circulating RSV-specific Abs with respect to matAb, or neutralization of infection leading to resolution of primary or re-infections, or enhanced disease in terms of the FI-RSV vaccine. Indeed, formalin treatment of RSV appeared to selectively obliterate the protective neutralization epitopes on the virus surface (Olmsted et al., 1986, Prince et al., 1986). An unbalanced immune response ensued in which the host developed high titres of F- and G-specific Abs lacking neutralizing activity, but formed immune complexes that may have contributed to exaggerated pathology following natural infection.

The mechanisms involved during neutralization would encompass the independent interaction of McAbs and the synergistic interaction of different McAbs (Anderson et al., 1988) coming

into interplay. During an investigation on the capacity of mixtures of anti-G McAbs to neutralize RSV infectivity, Martinez & Melero (1998) observed a reduction of virus titre (about 800 times) with a pool of Abs. Therefore, a single infection would stimulate partial protection, whilst successive infections, which would be reminiscent of the McAb pool or infant polyclonal sera, would result in enhanced or complete protection. The authors suggested that for a maximal effect to be seen in terms of a reduction of virus titres, this would need the contribution of Abs to the 3 epitopes identified on the G molecule working in synergy. Additionally, maximal synergy would be realized if these Abs do not compete for the same antigen binding site (Garcia-Barreno et al., 1989). It is therefore possible that the binding of several Ab molecules to the same G protein and the cross-protective epitopes on the F protein allows for complete protection.

Some investigators have found that G proteins were less able to stimulate neutralizing Abs or protective immunity than F protein (Table 3.6). Additionally, Anderson and others (1988) found that G McAbs gave only partial and enhanced neutralization, which perhaps they suggested, were less effective than complete neutralization afforded by F protein McAbs. However, it has also been observed that some subgroup specific anti-F Abs do exist and that they possess noticeably higher neutralizing activity than cross reactive Abs (West et al., 1994), despite the high levels of conservation noted in the F genes between the 2 subgroups of RSV. Mufson & Stanek (1996) also identified a group A variant that differed in its levels of antigenic reactivity to two anti-F McAbs generated against a group B strain. This implies that antigenic variation does occur not only in the G gene, but also in F.

Recently, a rapid molecular biology technique was used to screen large numbers of clinical samples from epidemics. The technique involved the reverse transcription polymerase chain

reaction (RT-PCR) of defined sections of both the G and N genes followed by restriction digestion analysis (RFLP). This therefore allowed for the investigation by molecular analysis of circulating strains, as well as allowing for an estimation of variability within an epidemic (Cane et al., 1991, Cane & Pringle, 1992, Sullender et al., 1991). Additionally, sequence data can be utilized in phylogenetic analysis of G gene nucleotide sequences, defining a number of genotypes or lineages of RSV within both groups (Cane et al., 1994, Cane & Pringle, 1992, Peret et al., 1998). However to date, the genotype nomenclature is not consistent between laboratories, and it is clear that new genotypes are continuing to emerge while others are no longer detectable (P. Cane, personal communications).

2.4.3 Relative prevalence of strains, hospital vs. community

The epidemiology of RSV disease is characterized by marked seasonal patterns (Chanock et al., 1970). RSV causes annual epidemics usually during the winter in temperate climates (Cane et al., 1994) although there are exceptions such as Finland where every 2 years a minor RSV peak has been observed in April followed by a major peak in December (Waris, 1991). In the tropics outbreaks have often been associated with the rainy season, although these epidemics tend to be less predictable in terms of date of occurrence (Cane & Pringle, 1991, Cane et al., 1999, Hazlett et al., 1988, Roca et al., 2001, Nokes et al., 2004, Venter et al., 2001, Weber et al., 1998). Additionally, Madhi et al., (2000) observed that HIV-positive children in South Africa show infection with RSV year round.

Extensive studies have been carried out on strain variability within epidemics, with most studies focusing on hospitalized infants. Thus, characteristic recurrent epidemics are observed through hospital surveillance (Cane, 2001). Outbreaks have been characterized as being abrupt in onset, the beginning usually being anticipated by the increased number of hospitalized

infants for bronchiolitis and/or pneumonia (Hall, 1999a). Epidemics have also been recorded in community and family study settings, and the link between the schooling siblings and the primary infant cases being shown to be strong (Hall et al., 1976, Weber et al., 1999). The mean duration of RSV epidemics was noted to vary, from 3.5 - 6 mo as noted from a 12-year surveillance in Chile (Avendano et al., 2003). Additionally, RSV is known to cause repeat infections throughout life although these re-infections are usually observed to be less severe and more often than not, are limited to the URT, especially in children older than 2 years. Intra-group antigenic variability may play a role in re-infections but as yet has not been fully defined (Sullender et al., 1998). Re-infections may be caused by either viruses from the same or different group (Mufson et al., 1987), and estimates of re-infection rates in children infected as infants varied from 74-83% in the second year and 46-65% in the third year of life (Glezen et al., 1986, Henderson et al., 1979). The exact role however, of re-infections in maintaining RSV transmission in communities together with its relationship with observed epidemics of primary infections remains unclear.

Both RSV groups and strains have been shown to circulate concurrently in individual epidemics (Hendry et al., 1989, Hendry et al., 1986). However, the relative proportions of groups vary from year to year (Hall et al., 1990, Hendry et al., 1989). The same genetic lineages have been shown to be present worldwide during the same period, as well, analysis of strains from successive epidemics showed that different lineages could predominate each epidemic, neither were all lineages present in every epidemic (Cane et al., 1994). Additionally, outbreaks caused by genetically distinct strains could follow an outbreak caused by a single strain (Hall, 1999b, Imaz et al., 2000, Walsh et al., 1997). It was observed that isolates of some lineages increased in numbers over several years and then declined. Cane and colleagues (1994) suggested that this was due to the herd immunity, a build up of resistance, in the

community to a particular genotype. In general though, group A strains are detected more often than group B (Cane et al., 1994, Cane & Pringle, 1992, Cane & Pringle, 1995, Cristina et al., 1991). Fluctuations in circulating strains from one site to the next may contribute to the variation seen in the severity of these annual outbreaks (Domachowske & Rosenberg, 1999).

Comparison of strains from hospitalized infants with those isolated from patients presenting to the out-patient department, that are thus representative of community circulating strains, showed that very similar viruses circulate in these 2 groups of patients (Zambon et al., 2001). Similarly, Venter and colleagues (Venter et al., 2002) conducted a study in South Africa in which they compared isolates from children hospitalized in Soweto with those from children attending rural community clinics. The authors observed that the same RSV strain could cause LRTIs, severe RSV-associated disease and mild URTIs in infants. Likewise, a rural community-based study in Kenya of a cohort of infants found similar results (Nokes et al., 2004, Scott et al., 2004).

However, less well defined is the influence of viral factors, which includes the effect of strain variation on severity. Some studies concluded that group A viruses causes more disease than B (Imaz et al., 2000, Walsh et al., 1997), yet in others, despite the observation of a predominance of group A RSV infection, there was no difference in severity between group A and B infections (McConnochie et al., 1990, McIntosh et al., 1993); and finally, others showed that group B infections were more severe than those by group A (Hornsleth et al., 1998). It is possible that certain viruses within an individual group are more virulent than others (Hall et al., 1990). These authors noted in a 15 year study period on RSV isolates from which 1209 hospitalized and ambulatory children were examined, strains from the different groups, denoted as A2 and B4, were more frequently found in hospitalized patients and A1 in

outpatients, whilst the two years with the highest rates of intensive care admissions, were those in which group A2 dominated. Differences in viral replication between the 2 groups have also been observed; group A viruses replicate to higher titres than group B viruses both in animals and *in vitro* cells cultures. Additionally, in many studies overall, group A viruses are isolated most frequently, despite group B strains predominating in some epidemics (Morgan et al., 1987, Scott et al., 2004).

2.5 Clinical Symptoms of RSV Disease

RSV has been shown to be a highly contagious pathogen, its transmission occurring through direct or close contact with contaminated secretions, droplets or fomites (Bricks, 2001). The agent survives on contaminated hands for more than a half hour, and thus, the latter is a common route of transmission for self-inoculation infections. It also survives for several hours on surfaces of contaminated objects (Mufson et al., 1991, Steinhoff et al., 1980). The organism then infects the respiratory tract mucosa. In addition, the conjunctiva can be affected. Symptoms subsequently appear following a 2-8 day incubation period, but commonly within 4-6 days (Johnson et al., 1961). If the infection is restricted to the upper airway, then the symptoms are similar to those of a severe URTI and includes cough, nasal congestion and commonly otitis media (Heikkinen et al., 1999, Monobe et al., 2003, Sagai et al., 2004).

The clinical picture of RSV infection however, varies according to age. Although RSV infection is rare in the first 4 weeks of life, epidemics in neonates have been identified (Hall et al., 1979). RSV infections were observed in 20-30% of babies studied in a neonatal unit. In this study, 61% of babies had a respiratory illness, and of these, approximately half had URTIs, whilst the remaining half experienced pneumonia. Moreover, from all viruses isolated in patients with pneumonia during the first month of life, 55% were identified as RSV (Hall et

al., 1979). The risk of serious infection in early life (1-3 months of age) when matAbs are present may lead to hospital admissions, which were observed to be as high as 1 in 40 in industrialized countries (Clarke et al., 1978). Consequently, the role of matAbs in protection against RSV disease is a subject of debate and remains a controversial issue as will be further examined in Chapter 3.

It has however been noted that severe life-threatening LRTD is most often seen during primary infection of 2-4 month old infants (Hemming et al., 1995) in whom airway infection takes the form of bronchitis, bronchiolitis (the most common RSV-associated disease), bronchopneumonia and/or laryngotracheitis. In immunocompromised individuals, the infection frequently (> 50% of infections) descends into the lower airways and progresses to give typical signs of LRTI (Wendt & Hertz, 1995, Whimbey et al., 1996). Additionally, both infants and immunosuppressed individuals have been shown to excrete large quantities of RSV in respiratory secretions for prolonged periods (Bricks, 2001). Several authors (Martinez, 2003, Sigurs et al., 2000) have suggested that RSV may predispose the infant to development of asthma later in life. Despite all the possible complications associated with RSV infection, the latter usually resolves in less than a week, although it tends to be more severe in children aged 8 to 30 weeks as mentioned above. Following RSV infection, solid immunity is not achieved and consequently, further infections occur throughout life (Hacking & Hull, 2002), but thereafter, the severity of illness decreases with increasing age, and especially more rapidly for bronchiolitis than for pneumonia (Parrott et al., 1973).

Various host factors have also been implicated in severe RSV disease during primary RSV infection. Foremost among these are young age (< 3 months), prematurity and the presence of

underlying cardiopulmonary or immunosuppressive conditions (Parrott et al., 1973). These have been listed in Table 2.2 below.

Table 2.2. Major risk factors for RSV infections (Englund, 1999, Englund et al., 1991, Glezen et al., 1981, Hall et al., 1979, Hall et al., 1986, Kaneko et al., 2001, Wang et al., 1995)

- Under one year of age, especially under 3 months of age
- Prematurity
- Presence of chronic pulmonary disease, particularly BPD and cystic fibrosis
- Presence of CHD, especially children with left-to-right shunt and pulmonary hypertension
- Cell immune deficiency; malignancies, chemotherapy, spinal cord or solid organ transplants
- Hospital admission during RSV seasonal outbreaks

In addition, other factors such as low social status, malnutrition, living in crowded environments (two or more individuals sharing the same bedroom, nursery attendance), birth within previous six months from the beginning of the RSV seasonal outbreaks, the mothers' low educational level, lack of breast feeding, and the domestic exposure to cigarette smoke have been considered and been said to act as risk factors predisposing to more severe disease (Glezen et al., 1981, Holberg et al., 1991, Law et al., 2002, Simoes, 1999, Simoes, 2003).

2.6 Treatment of RSV Disease

Medical intervention for the prevention (prophylaxis) and management (treatment) of RSV infections is basically supportive and involves close monitoring of the clinical evolution of the disease (Chavez-Bueno et al., 2005); additionally, nutritional status, oral hydration and ventilatory support are important (Chavez-Bueno et al., 2005, Simoes, 1999). Passive immunization with RSV hyperimmune immunoglobulin (RSV-IGIV) or Palivizumab, a neutralizing humanized mouse monoclonal Ab (95% human origin and 5% murine) directed against the F protein, is an alternative intervention method. Both however, are indicated in a

subgroup of selected high-risk infants and children – especially those who are premature, have BPD or CHD, and are usually administered in the months immediately preceding the RSV season (Groothuis et al., 1991, Hemming et al., 1987, Simoes et al., 1998). Usage of these agents as noted from these studies, showed a reduction in the incidence and duration of hospitalization due to RSV. On the other hand, with regards to Ribavirin, the only antiviral drug approved by the FDA, it is argued that this should be utilized early, as the greatest replication rate of RSV occurs up to 3 days following the onset of LRT symptoms (Bricks, 2001, Chavez-Bueno et al., 2005).

2.7 Diagnosis of RSV

Initially, viral isolation in conventional tissue culture was widely used to diagnose RSV infections, and has become the gold standard for laboratory diagnosis, despite results taking between 3-7 days, although this can range from 2-10 days (Piedra et al., 2002). Combinations of human epithelial cell lines have been used and include: human epithelial (HEp-2), human lung fibroblast (WI-38 or MRC-5), or rhesus monkey kidney cells which have been shown to be appropriate for recovery of RSV. As RSV is highly thermolabile, it requires stringent adherence to transport and storage guidelines, together with almost immediate inoculation of specimens in permissive cells for optimal recovery (Piedra et al., 2002). If this is not possible, the specimens need to be stored carefully to allow for recovery. Despite these shortcomings, cell culture still remains invaluable as it allows for the maintenance of a source for genetic analysis and antigenic change in virus populations as well as the discovery of new viruses. Many modifications to tissue culture exist such as the use of low-speed centrifugation in shell vial culture tubes that has been recently described (Matthey et al., 1992, Schirm et al., 1992).

Early detection of RSV infections is necessary to allow for early treatment such as the initiation of antiviral treatment with ribavirin and to prevent nosocomial transmission (Hall et al., 1985). Recently, it has become possible to do so using antigen-based rapid diagnostic methods. Previously, the radioimmunoassays (Sarkkinen et al., 1981) as well as the reverse passive haemagglutination assay (Cranage et al., 1981) were used as antigen detection methods and more recently new sensitive molecular techniques, RT-PCR (Coiras et al., 2003, Stockton et al., 1998) and real time PCR (Mentel et al., 2003, O'Shea & Cane, 2004) have been developed for the detection of RSV nucleic acid. However, the most popular methods of choice for detection of RSV antigen are immunofluorescence (IFAT) and rapid antigen tests especially in clinical settings. Both direct and indirect immunofluorescence techniques are available for the detection of RSV in exfoliated nasopharyngeal epithelial cells, with several kits being commercially available. Results of these tests are generally well accepted by clinicians due to their excellent sensitivities and specificities, the rapidity with which results are available, and their relative low costs (Englund et al., 1996). Furthermore, these techniques have helped provide an appreciation of how RSV spreads amongst hospitalized patients and has thus afforded an opportunity for early therapeutic intervention (Welliver, 1988). Specimens most frequently used for the detection of RSV from the respiratory tract have included throat swabs, nasal swabs, tracheal aspirates, nasopharyngeal washes and nasophyarangeal aspirates (Ahluwalia et al., 1987, Blanding et al., 1989, Englund et al., 1996, Hall & Douglas, 1975, Hughes et al., 1988, Stensballe et al., 2002).

In terms of serological detection methods, the complement fixation (CF) method has long been available for such diagnostic purposes. The membrane immunofluorescent antibody technique (MFAT) of Scott and others (Scott et al., 1976) enhanced serological diagnosis of RSV infection through the detection of IgG, IgA and IgM Abs. The ELISA has also been utilized

for the detection of human serum Abs (IgG, IgM and IgA) to RSV. It has been suggested that all methods of detection, viral antigen-based, isolation in cell culture or serologically-based detections should be undertaken in order to ensure maximal detection as a case missed by one method can be uncovered by another (Puthavathana et al., 1995).

In this project we utilized IFAT and RT-PCR to detect virus and ELISA to detect Ab as spelt out in Chapter 4, in order to diagnose RSV infection in a birth cohort.

2.8 RSV Vaccine Developments

For a vaccine to be effective against RSV, it most likely has to target the multiple strains of RSV that co-circulate during an outbreak. Moreover, a balance between a level of attenuation that is safe for infants and one with immunogenicity that is sufficient to protect against natural infection (Piedra, 2003) especially during early infancy should be sought. This is a time when immunological responsiveness is poor, and the presence of maternally transmitted Abs can exert a dampening effect upon infant immune responses (Murphy et al., 1988). Furthermore, the vaccine must not potentiate naturally occurring RSV disease, as was earlier observed with the formalin-inactivated RSV vaccine (FI-RSV) administered in the 1960s (Kim et al., 1969). FI-RSV was administered intramuscularly to infants and children, aged 2 months- 7 years. Unfortunately, the vaccine not only failed to protect against disease due to natural infections with RSV in naïve infants, but it induced an exaggerated clinical response; in addition, 2 infants who received FI-RSV died at 14 and 16 months following RSV infection (Kim et al., 1969). It has thus been suggested that the goal for vaccine design against RSV is not to prevent infection, but rather, to prevent virus-associated LRTI and complications (Durbin & Karron, 2003, Polack & Karron, 2004).

Various strategies have been employed in the search for RSV vaccines and include subunit vaccines, subunit vaccines combined with non-specific immune activating adjuvants, live attenuated vaccines, live attenuated genetically engineered vaccines and polypeptide vaccines (Karron & Ambrosino, 1998, Piedra, 2002, Piedra, 2003, Piedra et al., 1995). Of these, the purified F glycoprotein subunit (PFP) and live attenuated virus (cold passaged, temperature-sensitive mutants and genetically engineered strains) candidate vaccines are being evaluated clinically and may show promise with further development. As serious disease occurs in both high risk individuals who have previously experienced RSV infection, as well as in RSV-naïve infants, it seems likely that more than one type of RSV vaccine will be needed to immunize all those who would benefit from vaccination (Polack & Karron, 2004). To this end, a number of key groups have been identified for RSV vaccine programmes and these include young children, women of child-bearing age, as well as the elderly, although the rationale for vaccinating each group may differ slightly (Zambon, 1999). These vaccines are listed in Table 2.3.

Table 2.3. Research and Development Status of RSV vaccine development in human studies (adapted from (Kneyber & Kimpen, 2004, Maggon & Barik, 2004)

Candidate vaccine	Advantages	Disadvantages	Characteristics	Population evaluated	Vaccine status	Manufacturer
Replicating						
Live- attenuated traditionally derived	Mimics natural infection, nasal	Over-attenuation, under attenuation, loss of ts phenotype	*Cpts* (from RSV A) e.g. *Cpts* 248/955; *Cpts* 248/404	Adults, children, infants	Inactive	Wyeth/NIAID
					Phase I	Wyeth/NIAID
Recombinant technology	Combination with other vaccine type		*Cpts* 530/1009	Adults, elderly, children, infants		
Non-replicating						
Subunit purified fusion protein	Induction of neutralizing Abs; manufacturing controlled; safe in RSV-seropositive individuals	Possible disease enhancement; no $CD8^+$ T-cells induced	PFP-1/2/3: moderately immunogenic, but safe in RSV-seropositive patients; not explored in RSV-seronegative infants	Adults, children	Phase II/III (children with CF); Phase II (pregnant mothers)	Wyeth

Candidate vaccine	Advantages	Disadvantages	Characteristics	Population evaluated	Vaccine status	Manufacturer
BBG2Na peptide	Induction of neutralizing Abs; manufacturing controlled	Predisposition for Th2 skewed cytokine profile; no $CD8^+$ T-cells induced		Elderly	Inactive	Pierre Fabre
				Adults	Inactive	SmithKline Beecham
FG	Induction of neutralizing Abs	Disease enhancement		Adults	Phase II	Sanofi-Pasteur
F/G/M proteins (RSV A) subunit						
Others						
Live chimeric RSV/PIV-3/bovine PIV-3	Immunogenic, nasal				Preclinical	MedImmune
					Preclinical	Berna
Virosomes						

Note: *cp,* cold-passage; *ts*, temperature sensitive; CF, cystic fibrosis; PFP, purified F glycoprotein; NIAID, National Institutes

Protection against severe disease during the first months of life by the acquisition of passively acquired Abs, either through maternal immunization or natural infection, or alternatively by boosting humoral Abs through vaccination is of great importance. This project examines the rate of decay of matAbs, which has a bearing on the time of immunization as the latter have been said to impact negatively on immunization. The establishment of minimal protective thresholds in a developing country setting will further enhance our knowledge on expected levels that ought to be achieved following vaccination.

CHAPTER THREE

RSV Specific Maternal Antibodies

3.1 Introduction

A considerable body of evidence indicates that maternally derived Abs to the viral surface proteins - F and G, provide protection against serious lower respiratory tract infection in a majority of infants (Connors et al., 1991, Glezen et al., 1981, Ogilvie et al., 1981). It has been demonstrated that the presence of high levels of maternally derived Abs, as well as the administration of prophylactic intravenous immunoglobulin enriched for high levels of RSV neutralizing Abs (RSV-IGIV) or humanized monoclonal Ab against F protein have been shown to be associated with reduction of RSV-associated disease (Glezen et al., 1981, PREVENT, 1997, The IMpact-RSV Study Group, 1998). The use of maternal immunization to augment this protection in young infants against disease lends itself to further consideration. As there are few data on the role of factors such as co-morbidities, malnutrition and other risk factors on matAb levels within developing country settings, it therefore remains important to establish what the protective threshold would be. This would facilitate prediction of effective vaccination schedules. A schematic indicating the putative protective threshold with age following matAb decline is shown in Figure 3.1.

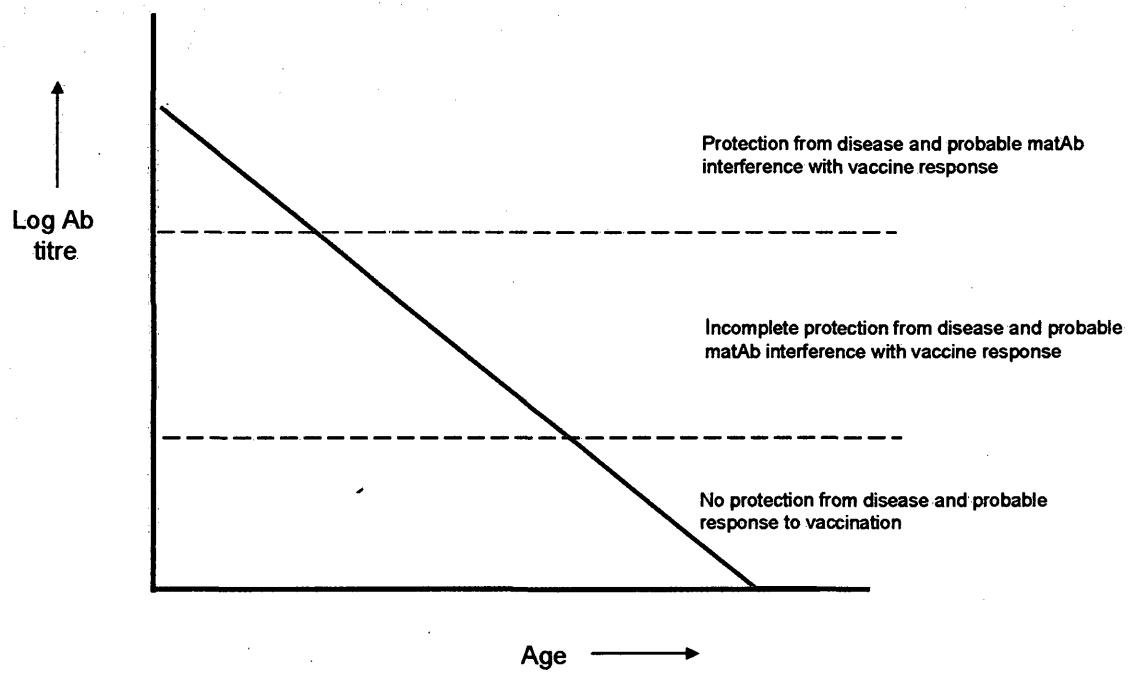


Figure 3.1. Proposed scheme of decline of maternal specific RSV Ab from birth depicting putative threshold levels of vaccine response.

A minority of otherwise healthy young infants have been observed to suffer RSV-associated disease. Additionally, acquired immunity to RSV remains incomplete resulting in individuals being susceptible to reinfection throughout life (Hall et al., 1991, Muelenaer et al., 1991, Wagner et al., 1989). It is plausible therefore that due to inadequate matAb levels and/or matAbs not being fully protective (strain-specific), this results in RSV disease under 6 months of age. The latter have also been said to interfere with the vaccine efficacy which in turn may lead to vaccine failure (Englund et al., 1998). The protective threshold against RSV infection is poorly defined. However, Piedra et al., (2003a) attempted to define this and reported it as being $\geq$6.0 $\log_2$ (95% CI 1.4 – 9.1) and $\geq$ 8.0 $\log_2$ (95% CI 1.1 – 7.7) against RSV associated-hospitalization for neutralizing Abs to RSV A and RSV B, respectively.

To estimate a baseline level for the protective threshold against RSV-associated disease, further investigation of convalescent phase Ab titres and/or Ab titres achieved in controls of

hospitalised patients not suffering severe RSV-associated disease but hospitalised at the same instance as cases (as similarly done in the Piedra study) should be carried out. This study will attempt to establish an immune correlate to RSV disease in infants from a developing nation, using convalescent titres of infants above 6 months who experienced RSV-associated disease. This information would be of importance for vaccine development.

This chapter provides an overview of RSV matAb acquisition and its role during the first year of life; in addition, primary RSV infection and hence early acquired immunity and re-infections in the child will be discussed.

3.2 Placental Transfer and the Decay of RSV-Specific Maternal Antibody

3.2.1 Literature search methods

A MEDLINE search of the literature from 1967 to 2006 was conducted using the National Library of Medicine and the National Institutes of Health's PubMed Services and Web of Knowledge on-line services. A review of the literature was conducted on passive immunity and placental exchange of RSV Abs. Keywords included: 'Maternal-Foetal Exchange', Respiratory Syncytial Viruses', 'Respiratory Syncytial Virus Infections', 'Immunity', 'Maternally-Acquired', 'decay maternal antibodies'. Except in a few instances, all searches were limited to the English language and humans. Furthermore, the reference lists from the selected retrieved articles were examined by searching the 'related articles' hyperlink to expand the search and thus identify other relevant literature. The articles found to be relevant are listed in Tables 3.1a and 3.1b.

Table 3.1a. Levels of RSV-specific matAb, maternal: cord blood titres

Country/ Reference	Number of samples	Assay	Maternal titres ($log_2 \pm$ sd)	Cord titres ($log_2 \pm$ sd)
Brazil (Cox et al., 1998)	39	ELISA	9.6 (0.05)*	10 (0.04)*
Canada (Le Saux et al., 2003)	761	ELISA-F	4.17 – 19.04	
Gambia, USA (Suara et al., 1996)	165 (Gambia) 218 (USA)	MNA	8.7 – 8.8 (±1.4) 7.9 – 8.8 (±1.5)	8.3 – 8.7 (± 1.4) 8.6 – 8.8 (± 1.6)
Netherlands (Brandenburg et al., 1997)	45	compELISA; VN		5.04 – 10.4
Netherlands (Heijtink et al., 1977)	200	CF	3.73	4.64
UK (Ogilvie et al., 1981)	130	MFAT	Control: 9.35 (± 0.23)* Com infected: 7.63 (± 0.31) Hsp infected: 7.43 (± 0.19)	
Poland (Wilczynski & Lukasik, 1994)	200	ELISA class; WB	IgG 1: 72% IgG 2: 22% IgG 3: 54% IgG 4: 48%	IgG 1: 83% IgG2: 18.5% IgG 3: 45% IgG 4: 48%
Turkey (Hacimustafaoglu et al., 2004)	49	ELISA	26 – 150 (± 29.8) RU/ml†	25 – 150 (± 26.8) RU/ml†
UK (Griffiths et al., 1982)	100	CF	3.27	4.24
UK (Nandapalan et al., 1986)	102	ELISA	0.7 – 12.6	
USA (Beem et al., 1964)	53	NA	1:4 – 1:64	< 1:4 – 1:64
USA (Bruhn & Yeager, 1977)	41	CF		Mildly ill: 3.6 Moderately ill: 4.0 Seriously ill: 4.1
USA (Glezen et al., 1981)	68	MNA		GMT: 62 – 97
USA (Kasel et al., 1987)	31	ELISA- F & G		F: 4.0 – 8.2 G: 4.0 – 7.7

MNA: microneutralization assay; CF: complement fixation assay; ELISA: enzyme linked immunoassay; VN assay: virus neutralization assay; NA: neutralization assay; MFAT: membrane fluorescence Ab technique; WB: western blot; anti-RSV, F- and G- are whole RSV, F-protein and G-protein respectively; com: community; Hsp: hospital

†: RU/ml – relative units; *: standard error of the mean

Table 3.1b. The prevalence of RSV-specific matAb, maternal: cord blood titres to specific RSV strains

Country/ Reference	Number of samples	Assay	Virus strain used as antigen	Maternal titres ($log_2 \pm$ sd)	Cord titres ($log_2 \pm$ sd)
UK (McGill et al., 2004)	20	MFAT	Infected		
			GA5(NCL/24882/96)	6.6 ± 0.86	3.6 ± 0.7
			GA5(NCL/15401/97)	6.5 ± 0.81	3.8 ± 0.74
			GA7(NCL/25137/96)	6.4 ± 0.85	3.8 ± 0.64
			GA7(NCL/17063/97)	6.3 ± 0.83	3.8 ± 0.74
			GA2/GA3(NCL/24702/96)	6.6 ± 0.92	3.8 ± 0.74
			GA2/GA3(NCL/8923/97)	6.7 ± 0.88	3.8 ± 0.74
			SAA1(NCL/2567/97)	6.5 ± 0.81	3.8 ± 0.74
			SAA1(NCL/21540/97)	6.4 ± 0.85	3.8 ± 0.74
			A2	4.5 ± 0.62	3.4 ± 0.88
			Uninfected		
			GA5(NCL/24882/96)	6.7 ± 0.66	5.6 ± 0.77
			GA5(NCL/15401/97)	6.6 ± 0.56	5.6 ± 0.94
			GA7(NCL/25137/96)	6.6 ± 0.54	5.5 ± 1.1
			GA7(NCL/17063/97)	6.6 ± 0.65	5.5 ± 1.1
			GA2/GA3(NCL/24702/96)	6.6 ± 0.56	5.3 ± 1.0
			GA2/GA3(NCL/8923/97)	6.5 ±0.65	5.3 ± 1.0
			SAA1(NCL/2567/97)	6.4 ± 0.46	5.4 ± 1.0
			SAA1(NCL/21540/97)	6.9 ± 0.59	5.3 ± 1.0
			A2	4.6 ± 0.54	3.3 ± 0.65

A further search using the key search terms 'Vaccines' AND 'Respiratory Syncytial Viruses' OR 'Respiratory Syncytial Virus Infections' OR 'Respiratory Syncytial Virus, human' being restricted to articles on studies on humans and in English, was undertaken and this resulted in 362 articles which was inclusive of 133 reviews. An additional search was also performed using the following combination of key terms: 'Immunization, Passive' OR 'Immunity, Maternally-Acquired' AND 'Respiratory Syncytial Viruses' OR 'Respiratory Syncytial Virus Infections' OR 'Respiratory Syncytial Virus, Human' AND 'Viral Vaccines', and again restricted to English and human studies, gave an additional 28 articles of which 19 were reviews. A final search using the "related articles" link to the study by Munoz et al., 2003 gave one extra article. Despite specifying a search for human based studies, the search engine also displayed studies on animal models. Immune responses to RSV vaccines will be discussed in section 3.6.

3.2.2 Placental transfer

MatAb transfer from mother to newborn is of the IgG class and is mediated by an active transport system via the Brambell Fcγ receptor localized to the placental membrane (Brambell, 1966, Gotlieb-Stematsky et al., 1983, Junghans & Anderson, 1996). Transfer occurs after the 28th week of pregnancy during the third trimester, the amount of matAbs in the foetal circulation increasing sharply thereafter, reaching maternal levels at around 35-40 weeks depending on IgG subclass (Madani & Heiner, 1989), and then exceeding maternal levels by a ratio of 1.2:1 to 1.8:1 (Kohler & Farr, 1966, Nicoara et al., 1999).

Few studies have measured RSV matAbs in both mother and their newborns. Usually the ratio of titres in cord or newborn blood to the titres in mothers is used as a common measure of placental transfer. It appears from Table 3.1a that cord blood titres are similar to or exceed

matAb titres on a case-by-case comparison, despite the various assays used. Additionally, infants admitted to hospital due to RSV infection, exhibited no apparent selective deficiency in matAb to viral glycoproteins of infecting virus strain or lineage (Table 3.1b). Due to methodological issues together with the small number of children and/or mothers sampled (Table 3.1a), this did not allow for a global ascertainment of the actual range of Ab titres transported across the placenta. This would be of great importance as it can be deduced that the highest levels of matAbs in the child would give protection against RSV-associated disease and hence give an indication of the putative protection threshold (Figure 3.1). Only one study made a comparison between maternal:cord blood titres between study populations in two geographically distinct areas, a developing country, Gambia, and a developed country, USA (Suara et al., 1996). As expected therefore from this mother-infant data, it can be assumed that cord blood samples from our Kilifi cohort will all have measurable titres, due to efficient transfer that approximates 1.0 (Suara et al., 1996) across the placental surface.

3.2.3 Risk factors for decreased placental transfer of RSV antibodies

There exist various factors that may influence RSV matAb transfer from mother to child including the following: maternal plasma concentration of Abs, gestational age, birth weight, nutritional status and co-infections such as malaria or HIV.

3.2.3.1 Maternal antibody concentration

It was noted that there existed seasonal variation in matAb titres, these being highest following a RSV season and have been reported to persist for 6 months (Le Saux et al., 2003, Nandapalan et al., 1986). Furthermore, higher titres were noted in mothers who had other children at home (Le Saux, 2003) perhaps due to frequent stimulation resulting from viral

spread from children to adults resulting in possible sub-clinical infections (Hall et al., 1976, Hall et al., 2001) and hence allowing for the boosting effects of the virus. As a result of active transport there is a preponderance for higher levels of virus-specific Ab on the foetal side of the circulation (Griffiths et al., 1982). Wilczynski and Lukasik (1994) suggested that there is a RSV IgG class specific transfer from mother to newborn, with this being most efficient for IgG 1, less so for IgG 2 and 3 and at similar levels for IgG 4. However, they mentioned that these observations were not statistically significant.

Additionally, and especially in developing countries due to poor sanitation, there are large numbers of infectious pathogens which all result in generation of Abs. This results in increasing amounts of the total maternal IgG and is associated with a lower fraction of maternal-specific IgG (Hartter et al., 2000) which is conserved across the placenta. A limitation in IgG transport may occur in such situations of hypergammaglobulinemia due to the limited number of available Fc receptors (Brambell, 1966). Nonetheless as noted from the study of Suara and others (1996), maternal: foetal transfer efficiencies were similar in the Gambia and USA.

3.2.3.2 HIV infection

In 2005, there was an estimated 17.3 million women living with HIV globally, with three-quarters or 13.2 million in sub-Saharan Africa. Around 59% of all adults in this region are women (http://data.unaids.org/pub/GlobalReport/2006/200605-FS_SubSaharanAfrica_en.pdf). In addition, the overall RSV case fatality rate in HIV-infected children have been described as being 12.7-fold greater than HIV-uninfected children, from studies carried out in South Africa (Madhi et al., 2006). RSV has been observed to cause increased morbidity and mortality in

immunocompromised children (Madhi et al., 2000, Madhi et al., 2001) under 2 years, and therefore it can be postulated that children born to HIV-infected mothers become susceptible to viral infection at a relatively early age. Recent studies describe the incidence of hospitalisation from RSV-LRTI as 45.0 per 1000 in HIV-infected, compared to 19.4 per 1000 in HIV-negative infants (Madhi et al., 2006). These newborns to HIV-infected mothers, may therefore display lower matAb levels as compared to their uninfected counterparts as noted for measles (de Moraes-Pinto et al., 1996, de Moraes-Pinto et al., 1993, de Moraes-Pinto et al., 1998). This may be due to a reduction in placental transfer of RSV-specific Ab as a result of immune complex formation that impairs transplacental IgG transport, the existence of defective IgG which may impair binding to the Fc receptor on trophoblasts, or a decrease in levels of Fc receptors during HIV infection (de Moraes-Pinto et al., 1993). The exact mechanism remains to be elucidated. To the best of our knowledge no data on the levels of RSV matAb in HIV-infected children have been published and thus it still remains unclear to what extent HIV infection influences RSV matAb levels.

There is also evidence to suggest that HIV infection increases the risk of prematurity and low birth weight (Leroy et al., 1998), both of which are potential risk factors for lower Ab transfer (see below for further details).

3.2.3.3 Gestational age/prematurity, birth weight and nutrition

A premature infant is born at less than 36 weeks gestation. Additionally, premature infants born small for gestation weight are even more compromised. Several factors predispose the premature infant to an increased severity of RSV-related illness. Two of the most important mechanisms are probably an inadequate immune response or defense against infection and

incomplete airway development (de Sierra et al., 1993). A term infant at birth has IgG levels approaching adult levels due to efficient matIgG transfer late in gestation, *i.e.* gestational age (GA) vary directly with the degree of placental transfer of Ab as the Brambell Fcγ receptor is expressed in the third trimester. This is not replicated in the preterm infant. Previous studies noted that immature infants displayed levels of anti-RSV that were significantly lower when compared to term infants (de Sierra et al., 1993) and as previously described for measles, tetanus toxoid, herpes simplex virus type 1 virus amongst other viruses (Okoko et al., 2002b, Okoko et al., 2001b, Wesumperuma et al., 1999). Low levels of RSV-specific Abs would therefore be expected in premature infants with higher frequencies of lower GA births observed in developing countries resulting in newborns starting with reduced amounts of Ab (Caceres et al., 2000) compared to their counterparts in the developed world. Madhi and colleagues (2006) observed that the burden of RSV-LRTI in children <32 weeks of GA in comparison to children born at 32-35 weeks GA was greater – incidence rates of 79.2 versus 34.2 (P=0.008) respectively, in a cohort of South African children followed over a 5-year period. These rates were similar to those reported in the developed countries (Boyce et al., 2000).

The effect of nutrition on cord blood titres has not been fully examined and remains unclear (Madico et al., 1996), although studies carried out to investigate the implication for vaccination schedules in light of the role of maternal measles Ab in rural Bangladeshi infants, have suggested that maternal nutritional status does not influence Ab level (de Francisco et al., 1998). Black (1982) however argues to the contrary and suggests that nutritional status affects immune status and may result in lower initial levels of matAb, which would thus imply that babies of these mothers would receive lower matAb during transplacental transfer.

3.2.3.4 Placental malaria

Maternal malaria results in the increased delivery of premature babies. Furthermore, the rate of low birth-weight babies is significantly higher if the placenta is parasitized by malaria (Okoko et al., 2002a), this frequency being higher for primigravidae. This occurs by parasite binding to chondriotin sulphate and other ligands located in the placenta thus resulting in premature and low birth weight babies (Menendez et al., 2000) and impairment of function, which may interfere with the beneficial effects of matAbs in various ways:

(a) Suppression of the immune response: Active malaria has been shown to suppress the immune response to some antigens (Duffy, 2003), including polysaccharides, and thus in pregnant women a similar mechanism implies that inadequate levels of matAb would accumulate on the maternal side and this would thus be reflected on the foetal side.

(b) Impairment of placental function: Malaria infection of the placenta impairs the transfer of Abs from mother to fetus (de Moraes-Pinto et al., 1998, Okoko et al., 2001a) as described for tetanus Abs (Brair et al., 1994) and would thus be expected to have similar effects on RSV matAb transport from mother to child.

3.2.4 Decay of RSV-specific matAb

Ab decay in the literature is reported as either biological half-life, this being the half-life before adjusting for the dilution effect of increased body weight, or as biochemical/catabolic half-life, which takes into account the dilution effect of increased body weight and is therefore a standardization method. For measles, mumps and rubella, clearance rates (biological half-life of matAbs) have been estimated to be between 35-40 days (Sato et al., 1979) or as long as 60 days for measles from studies performed in both the developed and developing nations (Caceres et al., 2000), and 51 (42-60) days for human parainfluenza type 3 (Lee et al., 2001).

From the various studies listed in Table 3.2, the decay of RSV-specific Abs appears to vary greatly from 26-100.3 days. However, no study reported 95% confidence intervals (CIs) for their estimates. While biological factors in some instance may account for the broad variation noted in these clearance rates, methodological issues and experimental design – longitudinal (which estimates the half-life in each individual), versus cross-sectional analysis (which estimates the mean half-life of the population) together with the small sample size used may also play a part.

Studies on several childhood infections spread by close contact, such as measles, mumps and rubella have also aided in the elucidation of virus transmission (Vyse et al., 2002). These viruses exist as single antigenic strains, with primary infections usually leading to solid immunity. This has allowed for simple models of transmission and statistical methods for analysis of Ab prevalence and mathematical models to aid design vaccination programmes (Brugha et al., 1996, Christie et al., 1990, Cutts & Vynnycky, 1999, Goncalves et al., 1999, Griffiths, 1974, Kebede et al., 2000). The half-life is useful in determining the duration of RSV matAbs in the infant, but does not give any information on whether infants are protected against RSV-associated disease, nor the age at which RSV matAbs will no longer interfere with vaccine responses. To this end, the proportion of children seroprevalent by age since birth is a much better indicator. A limitation of this approach is that it only provides an indirect measure of the decay rate. Eighty three to one hundred percent of newborns have been reported to be positive for RSV matAb, which has been noted to decline linearly with age (Table 3.3). Similar models have thus been used to estimate the duration for which RSV-specific Ab levels remain high in the population under study and this study will also estimate the probability of being seropositive over time and this is discussed further in section 3.3.

3.3 Models for Calculation of Mean Rate of matAb Decay

The biological decay rate, which represents an overall half-life (Lee et al., 2001) rather than the catabolic decay rate appears to be of greater importance especially if vaccine implementation is to be effective, as it allows for regional variation to be taken into consideration. For instance, it has been shown that in low-income developing countries protection by measles-specific matAb seems to decline faster in comparison to developed countries (Black, 1982, Black et al., 1986, Muller, 2001). Several factors could account for this difference and include income, which correlates with nutritional status which in turn affects immune status resulting in either increased matAb catabolism or lower initial levels (Black, 1982) and hypergammaglobulinemia (Muller, 2001).

Table 3.2. The mean duration of RSV matAb in infants.

Country, date of publication	Reference	Number of samples	Age range of participants (yr)	Study design	Test used	Half life (days)
Brazil, 1998	Cox et al.	115	0 – 40	Cross-sectional	ELISA	100.3*
Netherlands, 1997	Brandenburg et al.	45	0 – 6	Longitudinal	VN	26*
Turkey, 2004	Hacimustafaoglu et al.	49	0 – 6	Longitudinal	ELISA	91.2*
UK, 1983	Ward et al.	10	0 – 12	Longitudinal	RIPA	91.2
USA, 1964	Beem et al.	12	0 – 1.3	Longitudinal	NA	43*

ELISA: enzyme linked immunoassay; VN: virus neutralization assay; RIPA: radioimmunoprecipitation analysis; NA: neutralization assay; *: biological half life

Table 3.3. A selection of studies showing the proportion of infants between 0-12 months of age seropositive for RSV

Country, date of publication	Reference	Assay/Antigen tested	Proportion (%) of children with matAb during indicated month					
			0	1	3	6	9	>12
Brazil, 1998	Cox et al.	ELISA-anti-RSV	100	94	55	16		
Netherlands, 1997	Brandenburg et al.	comp-F ELISA	86		43	21		
		comp-G ELISA	91		58	21		
Turkey, 2004	Hacimustafaoglu et al.	ELISA-anti-RSV	83	73	6	2		
Japan, 2004	Ebihara et al.	NA	78				48*	54

ELISA-anti-RSV: enzyme linked immunoassay to whole RSV; comp-F or comp-G ELISA: competition ELISA for F and G protein respectively.
*this is an aggregate percentage of infants between 4-12 mo old

It is assumed that both matAbs and seroprevalence decay exponentially and constantly, and the mean rate of decay can be estimated from either parametric models (Cox et al., 1998, Hartter et al., 2000, Kebede et al., 2000, Lee et al., 2001) or linear regression on log Ab concentrations (Cox et al., 1998).

3.3.1 Model I: exponential decline model for maternal RSV Abs

The exponential change in Ab levels (y) with age (a) can be depicted as

$$dy/da = -\alpha y \qquad (1)$$

solving this, if a = 0, $y(0) = y_o$

and, if a> 0, $y_{(a)} = y_o e^{-\alpha a}$ (2)

Where, α is the rate of decay

y_o is the initial Ab level at birth

$y_{(a)}$ is obtained from the model, Ab titres at a given age, a

Taking logs on both sides of equation gives us our model:

$$\ln y_{(a)} = \ln y_o - \alpha a \qquad (3)$$

Where, $-\alpha$ is the slope, b of the line on the log scale and gives the rate of decay

and $\log y_o$ is the constant coefficient.

If the time taken for Ab to decay to half their initial value, $T_{1/2}$, then it follows from (3)

Then, $\ln y_o /2 = \ln y_o - \alpha T_{1/2}$

And $T_{1/2} = \ln(2)/ \alpha$ (4)

Also mean $D = 1/ \alpha$

median $D = \log y_o / - \alpha$ (5)

where D is the average duration of matAb protection

3.3.2 Model II: decay in seroprevalence

$$p(a) = p(0)(1-e^{-\delta t}) \quad (6)$$

Where,

p(a) is the proportion seropositive at age a

p(0) is the proportion seropositive at birth (a=0)

δ is the rate of decay

t is age

And $D = 1/\delta$ is the average duration of seropositivity

$T_{1/2} = \ln 2/ \delta$

It should be borne in mind that when calculating the cut-off point for seropositivity for the population under consideration, children with no detectable Ab (i.e. below the cut-off point for seropositivity) will influence the estimation of the decay rate. Furthermore, the inclusion of infected children in the calculation of $T_{1/2}$ will affect the value of this variable.

3.4 The Immune Response to RSV and the Implication for Protection by Maternal Antibodies

As mediators of disease may well differ between the lower and upper respiratory tract, quantitative data are required on the role of Abs or immune correlates to protection against RSV disease. The response to viral infection is complex; all facets of the immune system including Ab (secretory, serum and matAb), complement, mediators of inflammation, T and other types of lymphocytes and phagocytic cells (*e.g.* dendritic cells (DC), macrophages (mac) participate (Figure 3.2). Furthermore, host factors such as major histocompatibility complex (MHC) class I- and II-restricted cytotoxic cells (Brandenburg et al., 2001, Crowe, 2001, Openshaw, 2002, Welliver, 2003, Wright et al., 2002) work in concert to protect the child against or aid recovery from RSV-associated LRTI allowing for recovery from RSV infection. The prominence of one component over another *in vivo* or from one individual to the next may vary depending on the type of infection and on whether the host is limiting primary infection or resisting re-infection (Fishaut et al., 1980) with parasite factors also playing an important role. Following acute infection, the resulting immune response is characterized qualitatively and quantitatively by changes in specific Abs that include changes in immunoglobulin class distribution and Ab affinity (Meurman et al., 1992), protecting against URTI and LRTI. There also exists the possibility that breastfed children also benefit from matAbs in the form of colostrum (Bulkow et al., 2002, Downham et al., 1976, Holberg et al., 1991, Kristensen & Olsen, 2006).

Abs are described as the principle means of protection against diseases caused by viruses (Crowe, 2001). Virus-specific T cell responses, on the other hand, are involved in the clearance of RSV infection through the recognition of at least 6 major RSV proteins (N, M, SH, NS2, F and M2 - see Table 2.1) (Anderson et al., 1991; Cherrie et al., 1992). It is the

cytotoxic T-lymphocyte (CTL) through the recognition of MHC class I molecules that control infection by direct destruction of infected cells or by the release of antiviral cytokines (Russell & Ley, 2002). Despite this, it is argued that the F protein primarily induces a T_H1 type immune response, while the G protein induces primarily a T_H2 type response, contributing to both protective immunity and disease pathogenesis respectively (Alwan et al., 1993, Cannon et al., 1988, Connors et al., 1992, Jackson & Scott, 1996, Openshaw, 1995, Waris et al., 1996). These findings were also confirmed from studies using the BALB/c mouse model (Bright et al., 1995), which is often used as a model for the study of RSV infection. Children with defects in their cell-mediated immune response, as well as athymic or gamma-irradiated mice were observed to shed virus indefinitely (Cannon et al., 1987, Fishaut et al., 1980, Hall et al., 1986). Paradoxically however, a recent study described a novel role for the G protein showing that it was critical for the generation of CTL responses during infection independently of virus titres but dependent on the conserved cysteine-rich region (see Figure 3.2 below).

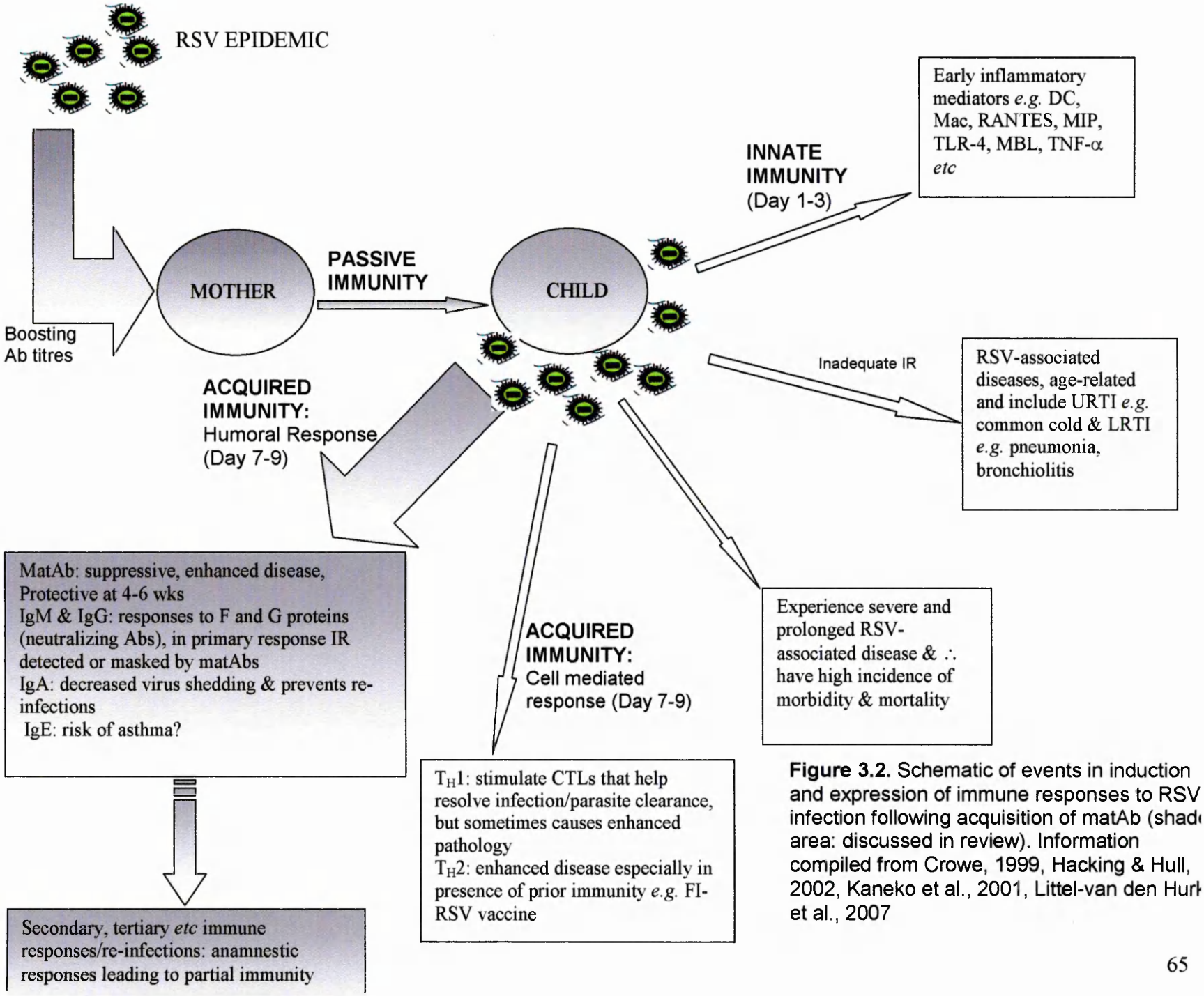


Figure 3.2. Schematic of events in induction and expression of immune responses to RSV infection following acquisition of matAb (shad area: discussed in review). Information compiled from Crowe, 1999, Hacking & Hull, 2002, Kaneko et al., 2001, Littel-van den Hurk et al., 2007

(Bukreyev et al., 2006). However, both the humoral and cell-mediated responses to RSV have been shown to be age dependent (Murphy et al., 1986a, Popow-Kraupp et al., 1989) initially being weak in the young. The role of matAbs on subsequent T- cell responses is conflicting. Bangham (1986) argues that passively acquired Abs to RSV can significantly inhibit the secondary cytotoxic T-cell responses in the mouse model, whilst Crowe et al. (2001) described a significant independent contribution by T cells (CD4+ and CD8+) to resistance to replication of RSV also in mice in the presence of passive Abs. The various facets of the immune response to RSV are shown in Figure 3.2.

The incidence of bronchiolitis in infants below 2 months of age has been observed to be markedly lower than in children 2 months and older (Parrott et al., 1973) as already mentioned. The incidence of pneumonia has also been documented as being lower but less strikingly so. Additionally, pneumonia is uncommon and bronchiolitis very rare in infants under 3 weeks of age (Hall et al., 1979, Neligan et al., 1970). Within this context therefore, matAbs play an important role in protection against severe RSV-associated illness. To further illustrate their importance in protection against disease, infants born during or just prior to the RSV season and hence experiencing low matAb titres have been described as being at maximum risk of admission to the hospital with RSV infection in the ensuing RSV season (Nandapalan et al., 1986). Moreover, the administration of passive polyclonal or monoclonal RSV-specific Abs to premature infants, who are at greatest risk of RSV-associated disease by virtue of having lower levels of matAb, prevents severe disease in this vulnerable population (Groothuis et al., 1993) if administered before the onset of infection. No protection was afforded by the latter when administered during acute infection (Collins & Pollard, 2002). These studies on passive transfer of neutralizing Abs demonstrate the beneficial role of

matAbs in offering infants protection; but the latter do not significantly affect viral replication in the URT (Brandenburg et al., 2001, Crowe, 2001, Wright et al., 2002). A number of studies have shown the relationship of neutralizing Ab titres to RSV with severity of LRTI, noting an inverse correlation between the two parameters (Glezen et al., 1981, Glezen et al., 1986, Lamprecht et al., 1976, Ogilvie et al., 1981, Roca et al., 2002, Ward et al., 1983). These observations underscore the importance of high matAb concentration in reducing the risk of severe disease in early months. However, severe disease is noted in children under 6 months of age at a time when matAb is virtually universal. Some clinical studies failed to find a relationship between the severity of illness and RSV Ab titre (Bruhn & Yeager, 1977, Wright et al., 2002). Furthermore, the results of the earlier vaccine trial with a formalin inactivated RSV vaccine, Lot 100, which led to the induction of high titres of RSV-specific Abs, however, still led to an exaggerated RSV-associated disease in the vaccinated group upon natural infection (Kapikian et al., 1969, Kim et al., 1969b). This group showed a worse prognosis in comparisons to the non-vaccinated group.

Several mechanisms in addition to the immature nature of the child's immune system may account for the occurrence of RSV-associated illness and will be further discussed later. The above evidence taken together is suggestive of the existence of a minimum protective threshold for matAbs against RSV-associated disease in the first few weeks of life, below which disease most likely occurs, and above which there is matAb interference with vaccine (refer to Figure 3.1).

3.5 Correlates of Immunity to RSV-Associated Disease - What is the Protective Immune Threshold?

The protective threshold against severe RSV-associated disease can be said to be the critical Ab titre that must be attained to ensure optimal protective efficacy against such disease. Ab titres include both matAbs and the child's own repertoire of neutralizing Abs stimulated upon primary infection. It can thus be argued that Abs measured in acute sera during primary infection therefore largely comprise matAb, whilst convalescent sera reflect both matAbs and the child's immune repertoire under 6 months. It has been shown that a low level of viral replication is important for an effective RSV immune response as inactivation of the virus with even minute quantities of Ab was sufficient to abolish immunogenicity of a candidate vaccine tested in the cotton rat model (Prince et al., 1982).

Therefore, it is important to define the matAb protective threshold as this impacts age of infection and hence, would aid in the appropriate timing of vaccination schedules. However, resolution of this threshold may be complicated by the fact that RSV undergoes antigenic variation. Moreover, the assay used to detect these isolates will also influence the quantity of Ab measured. For instance, Roca et al. (2003) observed higher titres to the locally circulating strain (Moz00) in comparison to the prototypes (A2 and 8/60) and even more so when the membrane fluorescence antibody technique (MFAT) was used in comparison to a neutralizing Ab assay. Each strain would therefore most likely give rise to its own threshold. This was recently described from studies carried out by Piedra and colleagues (2003a). The investigators noted that children with neutralizing Ab titres to RSV A of ≥ 6.0 $\log_2$ were 3.5 times less likely to have an RSV-associated hospitalization compared to children having titres below this value. Likewise, infants with neutralizing Ab titres ≥ 8.0 $\log_2$ to RSV B in

comparison to infants with titres below this level were 2.9 times less likely to experience an RSV-associated hospitalization. It is reasonable to assume therefore, that the determination of various thresholds arising from infection from different strains, would inform choice on the minimum protective threshold, irrespective of infecting strain, which should be attained by a vaccination regime. At this threshold, the child's own immune response may not be optimal – qualitatively (presence of immunoglobulins with correct avidity, optimal ratios of Ab classes e.g secretory Ab etc) and quantitatively, but the matAbs would nevertheless be protective against severe RSV-associated disease.

As mentioned above, it has been shown that high levels of matIgG protected against severe infection in the early months of life. One study in particular, on children, by Piedra and others (2003a) calculated 2 threshold values for infection by RSV A and B respectively. The authors therefore proposed minimal protective threshold values for Ab titres of ≥ 6.0 $\log_2$ (95% CI 1.4 – 9.1) and ≥ 8.0 $\log_2$ (95% CI 1.1 – 7.7) against RSVA and B respectively as correlates to immunity against RSV-associated hospitalization. Study participants with RSV-associated hospitalization were observed to have significantly lower GMT to RSV A (6.1 ± 2.9 versus 7.9 ± 2.4, $p < 0.001$) and RSV B (7.3 ± 3.2 versus 9.4 ± 2.3, $p < 0.001$) in comparison to controls.

It is difficult to compare studies due to variation in methods used. Kasel and others (1987) and Karron et al., (1999) had earlier attempted to similarly establish correlates of RSV immunity, but it is only the study by Piedra and others (2003a) that explicitly attempted to establish a minimal threshold serum Ab level. The former study found evidence for a protective effect against the occurrence of severe RSV illness to be associated with especially IgG anti-F. Pre-infection GMT against URTI being 8.1 ± 0.5 and 9.1 ± 0.77 against the G protein and F protein

respectively, whilst against lower respiratory disease, it was 6.4 ± 0.62 and 6.6±0.63 against G protein and F protein respectively. The assay used was an ELISA that was G and F specific, but no controls were defined. Additionally, the authors appear to be defining an URTI as a severe RSV illness. Karron and others (1999) noted that from the 219 children (≤ 6 months) who displayed Ab titres < 1200, this was strongly associated with the occurrence of severe disease (OR 6.2, p=0.03) using a rapid antigen EIA (enzyme immunoassay Test Pack) but a control group was not defined.

Table 3.4. A selection of studies comparing RSV-specific IgG titres in case/control studies

Reference	Sample #	Assay	Age (mo)	Comments	Ig G (log_2± sd)
Brandenburg et al., 1997	38	VN	< 6	control case	GMT: 51 GMT: 48
Glezen et al., 1981	68 cases & 575 controls	MNA	< 6	Pre-epidemic: control case Epidemic: control case	 GMT: 125 GMT: 97 GMT: 158 GMT: 62
Nielsen et al., 2003	1272 cases 6360 controls	ELISA	<24	control case	277 276
Piedra et al., 2003a	175	MNA	1 - 1068	control case	7.9±2.4 – 9.4±2.3 6.1± 2.9 – 7.3 ±3.2
Roca et al., 2002	62	MFAT	< 12	control case	GMT: 47.83 GMT: 10.94
Bulkow et al., 2002	175	MNA	<36	control case High-risk status control case	GMT: 266.9 GMT: 255.4 GMT: 357.5 GMT: 193.8

It is also possible that observed titres of controls in case/control studies (Table 3.4) as well as convalescent titres from studies investigating acute/convalescent titres would additionally aid the establishment of a minimum protective threshold titre. Ab titres of control were noted to be typically at higher levels than cases. Additionally, convalescent sera displayed more often than not, higher Ab levels than the respective acute sera, resulting in concomitant resolution of disease symptoms. Such studies either measure anti-RSV Abs (Table 3.5) or Abs to glycoprotein specific antigens (Table 3.6). As noted, in most instances, titres to the F protein were higher than those to G protein irrespective of the sample tested. These above findings nevertheless do not exclude a role for anti-G in host resistance to infection. From the latter studies therefore, it appears that the principal protective effects against RSV infection appear to be associated with IgG anti-F titres.

Age has also been shown to play a critical role in the determination of an individual's risk of hospitalisation (Glezen, 1977, Glezen et al., 1986, Henderson et al., 1979, Queiroz et al., 2002) and hence should be taken into consideration. In this regard, it was noted to primarily influence the immune response to F glycoprotein (Kasel et al., 1987, Murphy et al., 1986a, Murphy et al., 1986b, Welliver et al., 1989). A comparison of the convalescent titres from either sera or nasal pharyngeal secretions (NPS), from studies using the same assay and restricted to F glycoproteins gave a titre range from 5.4 –13.8 $\log_2$. This range encompasses the RSV A and B thresholds suggested by Piedra and others (2003a).

Table 3.5. A selection of studies comparing RSV-specific IgG titres in paired samples

Reference	Sample #	Assay	Study design	Age (mos)	Acute/conv sampling frame (days)	Comments	Ig G (log_2± sd)	
							acute	conv
Cranage & Gardner, 1980	14	IFA & CFT	x-section	< 1 - 8	30		3.4 ±1.6	5.8±1
Kim et al., 1969a	17	CF	x-section	2-40	3	2-6 7-12 13-24 >24	2.4 ± 0.6 2.0±0.0 3.2 ±2.7 2.0 ±0.0	3.6 ±0.9 4.8±1.5 6.4±1.7 5.0±1.7
Muelenaer et al., 1991	38	60% PRN	longitudinal	2-3		*Gp A RSV A RSV B *Gp B RSV A RSV B	 4.14 ± 0.33‡ 3.54 ±0.43‡ 3.43 ±0.1‡ 2.95 ±0.23‡	 6.91 ±0.4‡ 7.24±0.44‡ 4.55 ±0.42‡ 7.03 ±0.52‡
Nandapalan et al., 1984	16	ELISA		1- < 72 mos	14		8.4 ±4.3	10.2 ±3.5
Colocho Zelaya et al., 1994	42	ELISA	x-section	< 12	4		11.8 ±1.8	12.5 ± 1.6
Murphy et al., 1986b	18	60% PRN	X-section	4-21	161	RSV A (A2) 4-8 mos 9-21 mos RSV B (18537) 4-8 mos 9-21 mos	 5.8 ±0.3 3.7±0.2 6.2±0.5 3.8±0.3	 5.4±0.5 9.7±0.2 6.3±0.6 6.9±0.7
Murphy et al., 1986a	50	60% PRN	X-section	1-21	30-161	RSV A (A2) 1-8 mos 9-21 mos RSV B (18537) 1-8 mos 9-21 mos	 5.5 ±0.3 3.8±0.1 5.7±0.4 4.2±0.3	 6.5±0.4 9.0±0.4 7.4±0.5 8.0±0.4
Yamazaki et al., 1994a	22	ELISA – F & G (NPS)	x-section	0-16 mos	9 - 15	0 – < 6 6 – <16	4.3 ± 1.1‡ 1.8 ± 0.6‡	6.1 ±0.4‡ 8.6 ± 1.1‡

Reference	Sample #	Assay	Study design	Age (mos)	Acute/conv sampling frame (days)	Comments	Ig G ($log_2 \pm$ sd)	
							acute	conv
Wagner et al., 1989	31	ELISA	Longitudinal	< 48		*Long (RSV A) Gp A:IgG1 GpA: IgG2 GpA: IgG3 Gp B: IgG1 Gp B:IgG2 GpB: IgG3 *18537 (RSV B) Gp A:IgG1 GpA: IgG2 GpA: IgG3 Gp B: IgG1 Gp B:IgG2 GpB: IgG3	 3.6±0.2 5.0±0.3 4.3±0.4 4.6±0.5 6.2±0.6 4.0±0.3 3.7±0.3 4.3±0.3 3.7±0.3 3.3±0.0 4.6±0.7 4.2±0.5	 6.3±0.3 6.3±0.4 6.4±0.4 4.2±0.4 5.3±0.8 4.2±0.4 4.0±0.3 4.4±0.4 4.6±0.4 7.9±0.6 6.8±0.9 6.8±0.6
Yamazaki et al., 1994b	23	ELISA (NPS)	x-section	6 - 18	9 – 15	*Gp A Ag: Long Ag: 58-17B *Gp B Ag: Long Ag: 58-17B	 1.3 ± 0.4‡ 1.0± 0.0‡ 1.5± 0.5‡ 1.0±0.0‡	 8.1 ± 0.8‡ 7.1 ±0.7‡ 7.6 ±1.1‡ 8.6 ±0.6‡
Baumeister et al., 2003	22	IFA	x- section	1 - 23	14 – 21		GMT: 8	GMT: 589
Beem, 1967	11	NA	X-section	2-36			GMT: 2.4 ±1.7	GMT: 4.9 ±2.6
Brandenburg et al., 1997	38	VN	X-section	<6			GMT: 51	GMT: 75
Bruhn & Yeager, 1977	41	CF		1.8 – 3.3	33 – 36	mildly ill moderately ill seriously ill	GMT: 1:4 GMT: 1:5 GMT: 1:5	GMT: 1:27 GMT: 1:16 GMT: 1:30
Parrott et al., 1973	409		longitudinal	12- 121			GMT: 3.0	GMT: 30.1

Reference	Sample #	Assay	Study design	Age (mos)	Acute/conv sampling frame (days)	Comments	Ig G ($log_2 \pm$ sd)	
							acute	conv
Scott et al., 1984	28	MFAT	X-section	1-36	7-14		GMT: 24	96
Coates et al., 1963	22	NA	Longitudinal	1-14		Long 1-6 mo 7-14 mo CH 18537 1-6 mo 7-14 mo	 GMT: 2.1 ± 1 GMT: 1.8±0.2 GMT: 2.2±0.9 GMT: 2.2±0.8	 GMT: 5.3 ±1.3 GMT: 4.8±0.8 GMT: 6.5±2.1 GMT: 5.4±1.4
Fernald et al., 1983		MNA	Longitudinal	<12 -120		Control URTI-Asymptomatic URTI-Symptomatic LRTI	GMT: 64 GMT: 32 GMT: 16 GMT: 16	GMT: 48 GMT: 160 GMT: 160 GMT: 80
Meurman et al., 1992	93	ELISA	case-control	1 - 58	10 – 40	0 – 3mo 4 – 6 mo 7 – 12 mo 13 – 24 mo > 24 mo	GMT: 640 GMT: 240 GMT: 170 GMT: 190 GMT: 2380	GMT: 970 GMT: 1120 GMT: 2590 GMT: 4160 GMT: 10190

‡ denotes standard error of the mean

Table 3.6. A selection of studies comparing RSV-specific IgG responses to F and G glycoproteins in paired samples

Reference	Sample #	Assay	Study design	Age (mos)	Comments	Ig G (log_2± sd)			
						F protein		G protein	
						acute	conv	acute	conv
Piedra et al., 2003a	175	MNA	Case-control		control case	13.9 ±1.8 12.6 ± 2.6			
Kasel et al., 1987	34	ELISA-F & G	Longitu-dinal		< 6 mo 7 – 12 13 - 23	8.2 5.9 4.0	9.7 11.0 12.4	7.7 5.2 4.0	8.6 8.8 9.9
Mulenaer et al., 1991	38	ELISA- F & G	Longitu-dinal	2-3	A RSV A RSVB B RSV A RSVB	 5.02±0.62 4.00±0.74 5.22±0.46 5.33±1.00	 11.40±0.39 11.47±0.79 9.89±0.57 11.56±1.15	 4.21±0.66 4.25±1.04 4.89±0.59 4.89±1.03	 8.40±0.47 6.95±0.96 5.22±0.51 10.89±1.15
Toms et al., 1989	57	ELISA-F & G	x-section	<1- < 11		7.8±0.3	8.1±0.3	7.1±0.3	6.8±0.3
Murphy et al, 1986a	50	ELISA- F & G	X-section		1 – 8 mo 9 – 21 mo	9.6± 0.5 6.7 ±0.5	11.3 ±0.4 13.8± 0.3	9.5±0.6 6.7±0.5	10.9±0.4 13.2±0.3
Murphy et al., June 1986b	18	ELISA- F & G	x-section	4-21	4-8 9-21	10.6±0.6 5.1±0.8	11.3±0.4 14.3±0.3	10.6±0.6 5.7±1.1	10.3±0.6 13.7±0.5
Yamazaki et al., 1994b	23	ELISA F & G (NPS)	X-section	6-18	RSV A RSV B	1.0±0.0 1.0±0.0	6.2±0.9 5.4±0.9	1.0±0.0 1.0±0.0	4.5±0.9 1.2±0.4
Wagner et al., 1989	31	ELISA- F & G	Longitu-dinal	< 48	IgG1 IgG2 IgG3	4.9±0.3 5.7±0.4 4.8±0.4	9.4±0.4 7.0±0.4 8.3±0.5	3.7±0.1 4.8±0.4 4.2±0.3	6.7±0.4 6.5±0.5 6.6±0.3

3.6 Evaluation of the Induction of the RSV-Specific Immune Responses by RSV Vaccines

It is assumed that boosting matAb titres though either maternal immunization or the child's own immune repertoire through early childhood vaccination, should protect against severe disease. This would thus result in a decrease of severe RSV-associated disease, an increase in the age of primary disease as well as offering protection to infants below the age of 2 mos with underlying risk factors. It is therefore important to elucidate Ab titres and duration attained following vaccination to ascertain their effectiveness. Furthermore, this would further aid in the establishment of a minimum protective threshold against RSV disease.

Animal models have been used extensively to elucidate possible immune mechanisms to RSV infection. However, despite undergoing a series of laboratory and animal model evaluations, the immunogenicity of promising candidate vaccines do not always translate to humans. For example, the RSV *ts*-2 (temperature sensitive-2) mutant was seen to be overly attenuated and thus unsuitable for use in immunoprophylaxis of RSV illness as susceptible young children could not be uniformly infected with high dose of virus (Wright et al., 1982). Additionally, the same authors in a later study (Wright et al., 2000) also noted that following a second vaccine dose, this only led to a slight boost in immunity but did not lead to enhanced neutralizing Ab response in vaccine recipients as predicted by a chimpanzee model (Crowe et al., 1994). It was also demonstrated on numerous occassions that RSV vaccine candidates might retain sufficient residual virulence to cause mild symptomatic URTI or even enhancement of clinical disease in infants that was not predicted from animal models (Kapikian et al., 1969, Wright et al., 2000). Thus, this review will not consider animal models. Table 3.7 summarizes data from human vaccine studies.

Table 3.7. RSV-specific Ab responses between acute/convalescent or pre- and post-vaccination sera as a means of measuring the immune response to RSV infection

Author	Ab response to/ Dose	Subjects/ Selection bias	Age (mo)	Sampling time frame (days)	Comments	Antibody titres ± S.D.		% showing ≥ 4-fold increase
						Pre-vacc or acute sera	Post –vacc or convalescent sera	
1. Murphy et al., 1986	FI-RSV/ 0.5 mL (100-fold concentration)	69/ healthy or with bronchiolitis or pneumonia	2-40	90	Vaccinees (2-7 mo):			
					ELISA-F log_{10}	4.2 ±0.3	4.1 ±0.1	95[a]
					ELISA-G log_{10}	3.6 ±0.2	2.7 ±0.1	33
					Neut log_{10}	2.4 ±0.2	1.7 ±0.1	48
					Vaccinees (7-40 mo):			
					ELISA-F log_{10}	3.1 ±0.2	5.0 ±0.2	100
					ELISA-G log_{10}	2.2 ±0.3	3.6 ±0.1	93
					Neut log_{10}	1.6 ±0.1	2.2 ±0.1	60
					Natural infect (2-7 mo):			
					ELISA-F log_{10}	2.9 ±0.2	3.5 ±0.3	54
					ELISA-G log_{10}	2.8 ±0.3	3.5 ±0.1	64
					Neut log_{10}	1.7 ±0.2	2.4 ±0.2	64
					Natural infect (7-40 mo):			
					ELISA-F log_{10}	2.3 ±0.2	4.2 ±0.1	92
					ELISA-G log_{10}	2.2 ±0.2	3.7 ±0.1	92
					Neut log_{10}	1.2 ±0.1	2.8 ±0.1	92
2. Falsey et al., 1999		41/ healthy	< 600		Natural infect (N=11)			
					ELISA-F log_2	13.6 ± 2.0	16.4 ±1.4	
					ELISA- Ga log_2	12.0 ±1.5	14.1 ± 1.5	
					ELISA- Gb log_2	12.6 ±1.9	15.0 ±1.3	
					MNA log_2	10.7 ± 1.0	13.0 ±1.3	

Author	Ab response to/ Dose	Subjects/ Selection bias	Age (mo)	Sampling time frame (days)	Comments	Antibody titres ± S.D.		% showing ≥ 4-fold increase
						Pre-vacc or acute sera	Post –vacc or convalescent sera	
3. Murphy et al., 1988	FI- RSV/ 0.5 mL (100-fold concentration)	36/ healthy	2-21	90	ELISA-F log_2:			
					Vaccine	14.0 ± 0.9*	13.6 ± 0.3*	
					Natural infect.	7.4 ± 0.6*	12.9 ± 0.3*	
4. Belshe et al., 1982	Parenteral live RSV/ $10^{3.9}$ (0.5 mL) $TCID_{50}$	166/ healthy	6 -36	30	Vaccine GMT:			
					ELISA	< 50	204	
					Neut	< 2	48	
					CF	< 2	4.8	
					Natural infect GMT:			
					ELISA	90	2000	
					Neut	4	≥ 256	
					CF	< 2	18	
5. Wright et al., 2000	Live attenuated intranasal (*cpts* 248/404)/ 10^4 -10^5 pfu/mL	114/ healthy	1-59	30-90	Seropositive (15-59 mo):	14.1	13.9	0
					ELISA-F log_2	12.0	11.5	0
					ELISA-G log_2	9.3	8.8	0
					Neut log_2			
					Seronegative (6-24 mo):	8.1	12.5	71
					ELISA-F log_2	6.5	11.6	77
					ELISA-G log_2	3.3	7.3	68
					Neut log_2			
						9.8	10.2	78
					Infants (3-5 mo):	9.0	8.9	56
					ELISA-F log_2	5.0	5.8	44
					ELISA-G log_2			
					Neut log_2			
						14.7	13.2	0
					Infants (1-2 mo):	12.2	11.6	7
					ELISA-F log_2	5.9	4.4	0
					ELISA-G log_2			
					Neut log_2			

Author	Ab response to/ Dose	Subjects/ Selection bias	Age (mo)	Sampling time frame (days)	Comments	Antibody titres ± S.D.		% showing ≥ 4-fold increase
						Pre-vacc or acute sera	Post –vacc or convalescent sera	
6. Lee et al., 2004	Live nasal challenge/ 4.7 log_{10} $TCID_{50}$	65/ healthy	216- 540	28	ELISA-F log_2 ELISA-G log_2 MNA log_2	14.0 ± 1.5 13.3 ± 1.4 8.7 ± 1.0	16.0 ± 1.1 16.0 ± 1.2 10.0 ± 0.9	67 58 17
7. Piedra et al., 1996	PFP-2/ 50 µg (0.5 mL)	34/ CF	12-96	30-180‡	ELISA-F: 93.3% MNA: 66.7%	9.4 ± 3.1 6.3 ± 1.7 - 7.4 ± 1.5	≥ 4-fold rise ≥ 4-fold rise	
8. Munoz et al., 2003	PFP-2/ 50 µg or 0.5 mL	35/ healthy	Pregnant mothers		ELISA-F MNA		≥4-fold rise ≥4-fold rise	95 10
			Infants	0, 24, 180‡ 0, 24	ELISA-F MNA		≥4-fold rise ≥4-fold rise	
9. Piedra et al., 2003 b	PFP-3/ 30 µg protein + 0.5 mg $AlPO_4$ (0.5 mL)	294/ CF	12-144	28	ELISA F GMT log_2 ELISA G GMT log_2 MNA RSV A GMT log_2 MNA RSV B GMT log_2	13.1 ± 1.7 9.1 ± 1.6 5.4 ± 1.6 6.4 ± 2.4	17.9 ± 1.2 9.3 ± 1.6 7.9 ± 1.8 8.4 ± 1.9	97 3 67 55
10. Gonzalez et al., 2000	Combined live attenuated *cpts* 248/404 & PFP-2/ 25 µg protein + 0.5 mg $Al(OH)_3$ (0.5 mL) & 107 pfu/ mL *cpts*	60/ healthy	216-480 (18-40 yr)	28-35	Young (216- 480 mo) A†: ELISA F log_2 ELISA G log_2 MNA RSVAlog_2 Young (216- 480 mo) B^: ELISA F log_2 ELISA G log_2 MNA RSVA log_2	 13.9 13.1 9.9 14.4 13.4 9.9	 14.0 13.4 10.1 16.4 13.4 10.6	 92 8 28 60 0 16

Author	Ab response to/ Dose	Subjects/ Selection bias	Age (mo)	Sampling time frame (days)	Comments	Antibody titres ± S.D.		% showing ≥ 4-fold increase
						Pre-vacc or acute sera	Post –vacc or convalescent sera	
11. Groothuis et al., 1998	PFP-2 / 50 µg (0.5 mL)	21/ with broncho-pulmonary dysplasia	> 12	30	ELISA F log_2 MNA RSV A log_2	15.0 ± 2.0 6.5 ±3.6	20.0 ± 1.1 8.78 ± 1.6	
12. Power et al., 2001	BBG2Na/ 10, 100 or 300 µg	108/ healthy	216-540 (18-45 yr)	30	ELISA (AU)# 10 µg VN (AU)# GM, 10 µg ELISA (AU)# 100 µg VN (AU)# GM 100 µg ELISA (AU)# 300 µg VN (AU)# GM 300 µg	145 265 167 174 186 248	228 318 445 310 616 432	

Author	Ab response to/ Dose	Subjects/ Selection bias	Age (mo)	Sampling time frame (days)	Comments	Antibody titres ± S.D. Pre-vacc or acute sera	Antibody titres ± S.D. Post –vacc or convalescent sera	% showing ≥ 4-fold increase
13. Wright et al., 1982	Highly attenuated live vaccine, *ts*-2/ $10^{6.3}$ pfu/mL (0.5 mL)	29/ healthy	6-60		6 mo: ELISA Neut 8 mo: ELISA Neut 9 mo: ELISA Neut 11 mo: ELISA Neut 12 mo: ELISA Neut 14 mo: ELISA Neut 24 mo: ELISA Neut	68 12 < 50 < 4 < 50 < 4 < 50 < 4 < 50 4 < 50 4 < 50 < 16	50 5 < 50 < 4 < 50 6 709 13 < 50 5 < 50 15 < 50 < 16	
					14 mo: ELISA Neut 21 mo: ELISA Neut	< 50 < 4 < 50 < 4	3, 092 350 3, 046 110	
					~~Subsequent natural~~ infection URI, mild wheezing 9 mo (ELISA) 11 mo (ELISA) URI, bronchiolitis	< 50 60	6,318 8,588	

					8 mo (ELISA)	< 50	75	
					URI 6 mo (ELISA)	< 50	203	
					Undetermined 14 mo (Neut)	4	100	
14. Karron et al., 2005	Recombinant live attenuated vaccine, rA2*cp*248/404ΔSH & rA2*cp*248/404/1030ΔSH/ $10^{4.3}$-$10^{5.3}$ pfu/ mL (0.5 mL)	31 adults; 95 children; 44 infants/ healthy	1-2 mo, ≥6 mo	30-60	Adults: rA2*cp*248/404ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	14.1 ± 0.8 13.4 ± 1.3	14.1 ± 0.8 13.4 ± 1.1 8.6 ± 1.1	0 0 0
					rA2*cp*248/404/1030ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	8.5 ± 1.0 14.3 ± 1.2	14.2 ± 1.3 13.1 ± 1.9 7.9 ± 0.6	0 6 0
					Children seropositive: rA2*cp*248/404ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	13.0 ± 1.5 7.9 ± 0.7	15.1 ± 1.4 12.3 ± 1.2 8.0 ± 1.9	0 0 0
					rA2*cp*248/404/1030ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	15.3 ± 0.9 12.4 ± 0.9 7.8 ± 1.7	15.0 ± 1.3 12.0 ± 1.5 8.6 ± 1.3	0 8 0
					Children seronegative: rA2*cp*248/404ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	15.1 ± 1.6 12.2 ± 1.9	13.3 ± 1.6 11.7 ± 1.1 6.6 ± 1.5	88 88 88
					rA2*cp*248/404/1030ΔSH ELISA F log_2	8.6 ± 1.4	10.9 ± 4.2 8.9 ± 3.2 5.2 ± 2.6	36 36 33

					ELISA G log_2 Neut log_2			
						9.1 ± 2.7	12.7 ± 1.4 12.4 ± 1.5	0 0
					Infants: rA2*cp*248/404ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	7.0 ± 2.2 3.3 ± 0.0	7.0 ± 1.1 12.5 ± 1.4 12.1 ± 1.2 6.8 ± 1.6	0 0 0 0 0
					rA2*cp*248/404/1030ΔSH ELISA F log_2 ELISA G log_2 Neut log_2	7.7 ± 3.0 6.6 ± 2.0 3.4 ± 0.3		
						13.6 ± 1.2 13.3 ± 1.6 7.7 ± 1.0		
						13.4 ± 1.3 12.7 ± 1.1 7.4 ± 1.7		

* :S.E.; Vacc: vaccination; Infect. infection; GMT: geometric mean titres; NEUT: neutralizing assay; MNA: microneutralizing assay;(AU)#:arbitrary units; ELISA-F: F specific ELISA; FI- RSV: formalin-inactivated RSV vaccine; PFP: purified fusion protein; *cpts*: cold-passaged temperature sensitive; ‡: end of RSV season; **A**† : vaccine regime of *cpts* 248/404 + PFP-2 sequentially; **B**^: vaccine regime of *cpts* 248/404 + PFP-2 simultaneously. [ ] indicates natural RSV introduced into group 3 days before vaccine administration.

Note: For all the above studies, where a sero-conversion was noted post-vaccination or for convalescent levels, the Ab titres attained were higher than those noted for placebo controls. However, for the 1st study listed in this table, 1. Murphy et al., 1986, it is not clear how the authors calculated that 95% infants, indicated as [a], showed ≥ 4-fold increase, when the figures show a decline in Ab titres between pre- and post-vaccination.

From the majority of the above studies, seroconversion occurred in vaccine recipients. Despite this, these responses never attained the same magnitude as that seen following natural infection. These titres seen post-vaccination are similar to those observed in Tables 3.4-3.6. Again, it is observed that younger vaccinees, aged between 1-6 mos, did not develop a robust immune response to the G protein as do older vaccinees. The mean Ab titres of anti-F IgG Ab were seen to be 4-fold or higher than placebo recipients, with seroconversion occurring from between 54-97% of recipients. However, neutralizing Ab responses were seen to be modest or significantly lower in comparison to F Ab levels, despite the development of high-level F Ab responses. It has also been argued that the inability to infect seropositive children even though challenged with live attenuated RSV vaccines is indicative of RSV immunity (Wright et al., 2000). Additionally, in some instances, upon natural RSV infection post-vaccination, a significant rise in Ab titres was not realized. It is thought that the stimulated vaccine-specific Ab titres may be of sufficient magnitude, that an additional increase in boosting titres is not realized on subsequent natural RSV infection (Piedra et al., 2003b). This is thus suggestive of a minimal protective threshold allowing for the protection of infants from serious RSV-associated illnesses.

For the young children who failed to respond to vaccination, the authors account this to the presence of circulating matAbs, which in turn prevented Ab stimulation. From 4 studies (7, 8, 9, and 11) and an additional 3 studies (1, 3 and 10) listed in Table 3.7 that monitored RSV-specific Ab titres to the end of the RSV season and for a period of 2-4 mo post-vaccination (Figure 3.3) respectively, high Ab titres were still realized by the end of the season.

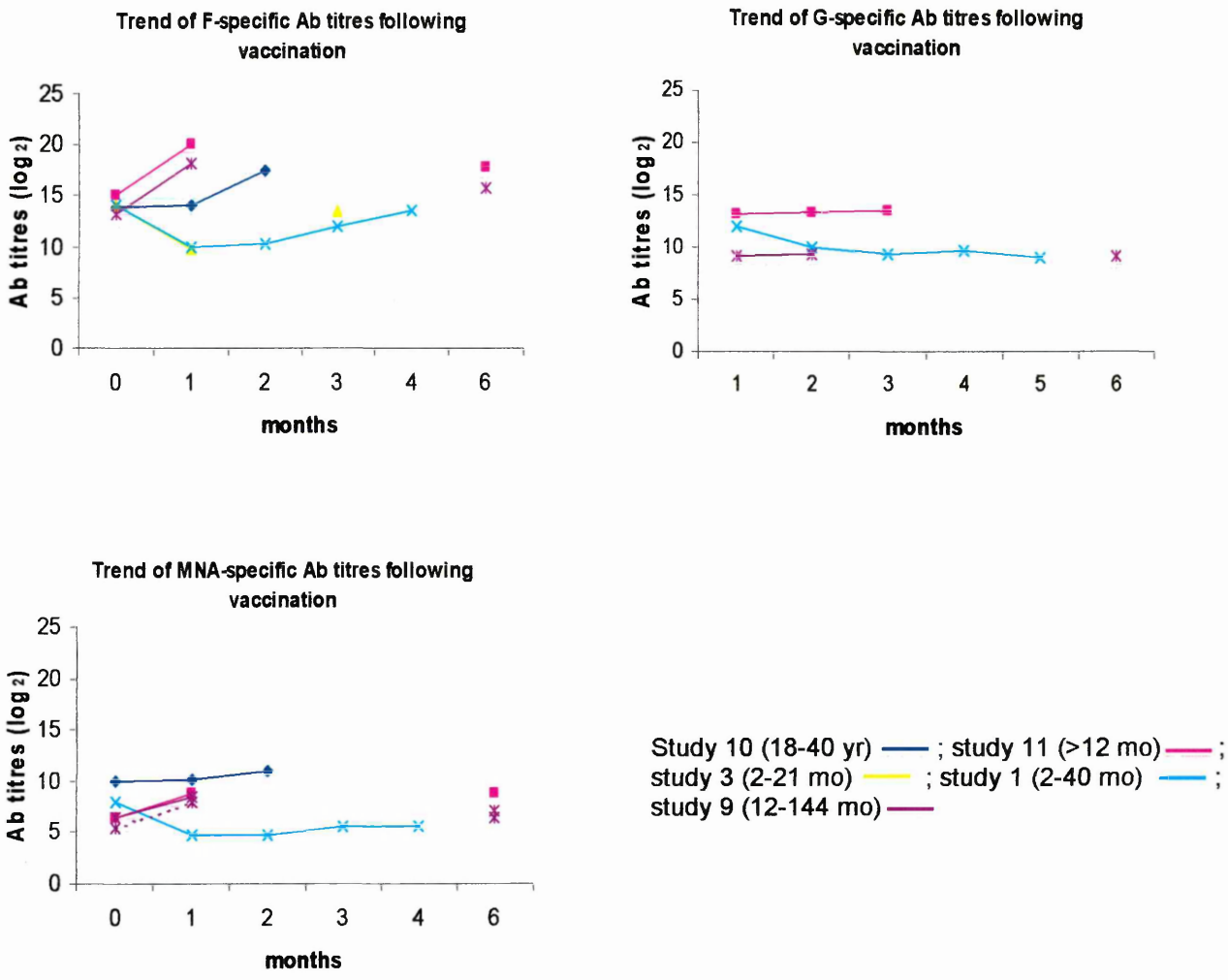


Figure 3.3. Trends of Ab levels following vaccination over a period of time (see Table 3.7 for details and references)

Consequently, the above studies on PFP and *cpts* in some instances, showed either no increase in frequency or severity of RSV-associated illness, or, a reduction in respiratory disease morbidity during the entire RSV season in infants of vaccine recipients when compared with controls and as such conferred protection against RSV-associated illnesses to young vaccinees. With regards to adults, this implies that as they are able to maintain high Ab titres throughout the RSV season, maternal immunization lends itself to further investigation, with a more immunogenic vaccine having the potential of resulting in higher serum and breast milk Abs against RSV in infants of vaccinated mothers (Munoz et al., 2003). On the other hand,

vaccinees who were infected with RSV were observed to experience significantly fewer ALRTIs (Piedra et al., 1998, Wright et al., 2000), required fewer courses of antibiotics and had fewer days of illness during the RSV season and this was especially so in children with underlying conditions such as cystic fibrosis and bronchopulmonary dysplasia, in comparison to RSV-infected controls (Groothuis et al., 1998, Piedra et al., 1996).

Munoz and colleagues (2003) from their observations postulated that transplacental transfer of RSV specific anti-F IgG as well as neutralizing Abs to both RSV A and B remained efficient, being greater than 100% in the case of anti-F IgG. Furthermore they calculated that for all vaccine recipients who initially began with low pre-vaccination titres, were able to achieve GMT of neutralizing Abs to both RSV A and B of > 6.0 Log_2. This is equivalent to the protective threshold value calculated by Piedra and others (2003a) as mentioned above. Another study (Gonzalez et al., 2000) observed that vaccine response rates (ELISA and neutralizing Ab titres) correlated with pre-vaccination titres, not age. They postulated that the diminished immune responses to vaccines may be as a result from the levels of pre-existing Abs rather than immunosenescence. Previous studies (Falsey & Walsh, 1998, Falsey et al., 1999, Walsh & Falsey, 2004a) have noted that older adults (≥ 65 years) with low titres of Nt Abs may be at greater risk of developing symptomatic RSV infections in comparison to those who display higher Ab titres. Moreover, their immune response to RSV infection can be as vigorous as younger subjects (18-40 years). It is therefore plausible as mothers possess varying titres of RSV-specific Ab titres, their infants will receive varying titres of matAb. It is consequently important to resolve what the critical protective threshold ought to be, above which pre-existing Abs will suppress the child's own immune response leading to risk of

infection and similarly, below which the child remains at risk of infection in the subsequent RSV season.

Vaccine efficacy may be predicted by serological correlates of protection based on the level of Ab achieved by the immunized population (Siber, 1997), which is also affected by the decay rate of Abs. One study (Piedra et al., 2003a) calculated the rate of decay of monthly GMTs for both Nt and ELISA binding Abs as occurring at approximately 0.25 log2 per month, implying protective levels are short-lived. Neutralizing Abs appear to be a more accurate predictor of infectability (Lee et al., 2004) as these responses are to both RSV groups A and B, these are only ~50% related through their G gene amino acid sequence and only 5% related antigenically (Johnson et al., 1987). As both decay at similar rates this needs to be taken into consideration when calculating the protective threshold. Additionally, it should be remembered that Ab concentrations achieved following immunization are not always absolute predictors of individual protection although they allow for the estimation of the probability of protection in the individual or in a population (Siber, 1997).

Therefore, as matAb are protective for a limited period after birth, the susceptible infant population includes those infants whose initial level of passively acquired Ab is low, as well, infants old enough at the time of RSV season who had their levels fall below the protective threshold levels. As a result, this group of infants are uniquely susceptible as they lack both cross-reactive immunity from prior exposure and passive protection (Puck et al., 1980). Despite the short-lived nature of protection, it remains important to establish immune correlates which might be complicated by such factors as presence of matAb to high decay rate, as this is not only critical for vaccine development, but allow prediction of vaccine

efficacy in populations other than those in whom efficacy was initially demonstrated (Siber, 1997).

3.7 The Suppression of the Infant's Immune Response by matAb

Suppression of infant Ab response by matAb to the virus may occur if, for instance, antigenic load happens to be sub-optimal as a result of these circulating Abs partially suppressing viral replication (Watt et al., 1986). Thus, the infant's own immune system is ineffectively primed with no Ab response. Above a certain protective threshold, matAb could suppress the infant's immune response accounting for RSV disease in the presence of matAb. It has been observed in experimental animals that the immune response is suppressed on administration of passive Abs (Belshe et al., 1982, Prince et al., 1982). Alternatively, the child's immune system could still be stimulated; however, the Abs that result may not be associated with appreciable neutralizing activity (Murphy et al., 1988). In other words suppression by matAb results in either suppression of the total Ab response (quantitative response) or is selective for epitopes (qualitative) involved in induction of neutralizing Abs (Murphy et al., 1988). Qualitative changes also embrace changes in the IgG class distribution as well as affinity of Abs (Meurman et al., 1992).

3.8 Other Immune Responses and Factors Affecting them

The inability of the infant to develop a solid protective immune response following primary RSV infections and re-infections remains unexplained, but the percentage of RSV-associated hospitalizations has been observed to decrease with age. Piedra and others (2003a) noted that 40.7%, 38.1% and 11.8% of children with LRTI in age groups <1 year, 1 – 4 years and ≥ 5 years respectively were RSV-associated hospitalizations. Similar trends were noted in other

studies with a decreased frequency of lower respiratory involvement, primarily a decrease in bronchiolitis, being associated with increasing age (Fernald et al., 1983, Glezen et al., 1986, Hall et al., 1991, Henderson et al., 1979). So, immunity induced following a single infection has no demonstrable effect on RSV illness associated with re-infection a year later, but a considerable reduction in severity of third infection was observed (Glezen et al., 1986, Kawasaki et al., 2004) The mechanism for mildness of clinical features at re-infection has been attributed to maturation of the respiratory system and immunity with age and the high acquisition of immunity after primary RSV infection (Kawasaki et al., 2004). The authors suggested an inverse relationship between neutralizing Ab levels and illness severity.

Protection against re-infection with RSV is mainly conferred by both serum (IgG-discussed above) and mucosal (IgA) Abs (Domachowske & Rosenberg, 1999). IgA is the major Ab isotype of respiratory tract secretions and is hence targeted to mucosal surfaces (Kerr, 1990). As a result, this immunoglobulin can bind and neutralize viral proteins intracellularly at the site of initial replication in the epithelial cells thus having an important protective role against infection (Fisher et al., 1999, Kaul et al., 1981, Tsutsumi et al., 1995) In this regard, the stimulation of IgA would play an important role against RSV, a mucosally restricted pathogen. Indeed, multivariate analysis found that low RSV-specific nasal IgA was an independently significant risk factor for RSV infection (Walsh & Falsey, 2004b). Indeed, several studies have demonstrated that viral shedding stops coincident with the emergence of secretory IgA (McIntosh et al., 1978, McIntosh et al., 1979, Mills et al., 1971). This local immunity thus appears to be the main mechanism responsible for the resistance to RSV in the URTI (Olmsted et al., 1986). Nonetheless, it has been suggested that matAb also affects IgA response during

primary infection (Yamazaki et al., 1994a), and it remains important to establish the minimal threshold level for optimal immune response.

3.9 Summary and Conclusion

MatAb of the IgG class is transferred to newborn from mother during gestation. At birth, cord titres can equal, exceed or be at levels that are lower than maternal titres. Various risk factors have been noted to decrease placental transfer, otherwise, transfer is efficient and approximates a maternal:cord titres of 1.0. RSV clearance rates were observed to vary from 26-100.3 days, whilst the proportion of children with RSV-specific matAb decreased as children got older. In one study, by 6 months of age, the proportion of children with matAbs was 2%.

The role of matAbs as immune correlates of protection against RSV-associated disease in early childhood remains controversial. There possibly exists a protective immune threshold above which matAb may interfere with vaccine efficacy as well as cause suppression of the infant's immune response. Additionally, below this level both vaccines and the child's immune response would be optimal.

CHAPTER FOUR

Materials and Methods

4.1 Study Site

4.1.1 Demographic features

These studies were carried out at the Kenya Medical Research Institute (KEMRI)/Centre for Geographical Medicine Research-Coast (CGMRC), Kilifi, situated at the Kilifi District Hospital (KDH) in Kilifi District, an area of 4779 km^2 (Central Bureau of Statistics, 2001) in rural coastal Kenya. The population of the district at the last national census was 544, 303 (Central Bureau of Statistics, 2001; http://www.statoids.com/yke.html).

KDH is situated 60 kilometres north of Mombasa on the Kenyan Coast and serves people living in the two areas separated by the Kilifi Creek. The population is predominantly Giriama, a subgroup of the main ethnic group Miji Kenda that consists of nine closely related subgroups. An area, including Kilifi township extending 40 km along the Indian Ocean between 2 creeks and 30 km inland was defined in 1991 for intensive epidemiological-demographic surveillance (EPI-DSS, Figure 4.1) and has been the base for several epidemiological, public health, bed-nets and drug intervention research projects (Snow et al., 1993). The population figure of the Epi-DSS area is estimated to be 213, 152 (Round 4 census, August-November, 2003).

The total population in Kilifi district comprises approximately 45% of people ≤ 14 years, with children under the age of 5 comprising 18% (Central Bureau of Statistics, 2001; http://www.statoids.com/yke.html). More than 80% of paediatric admissions to KDH originate

from the demographic surveillance area. In 1999, infant mortality for the district was 85.3 deaths /1000 births (English et al., 2003).

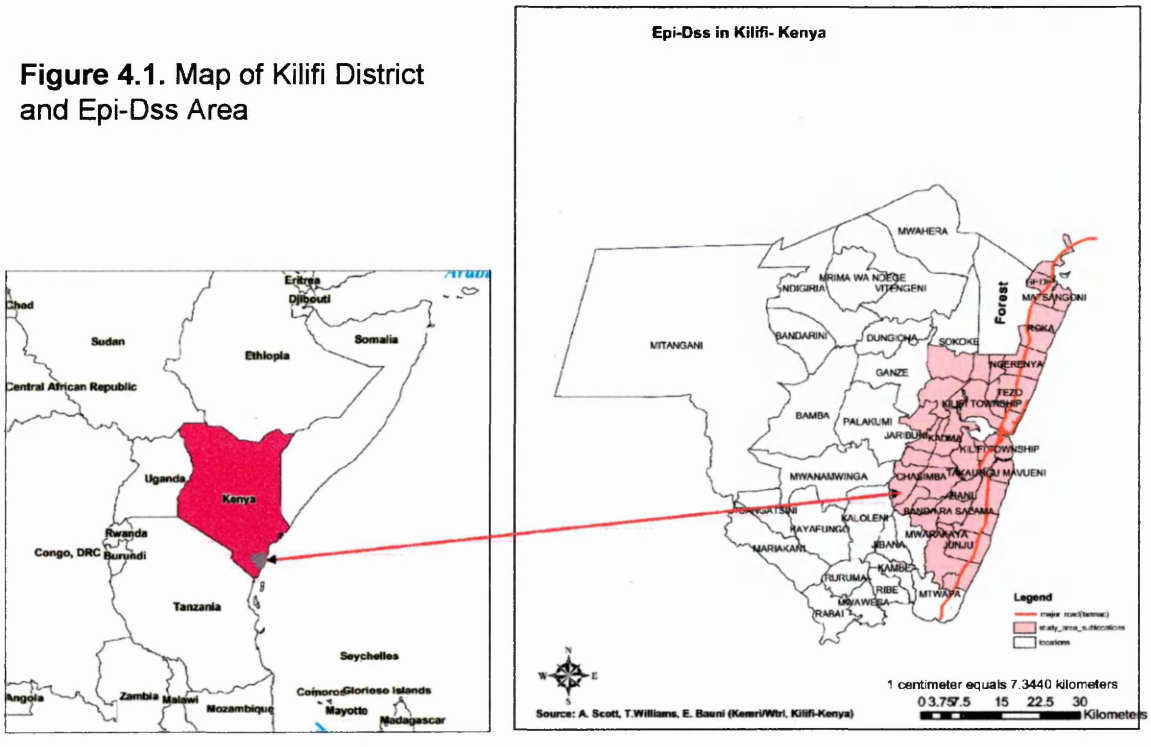


Figure 4.1. Map of Kilifi District and Epi-Dss Area

Subsistence farming is the main occupation, with farmers growing maize, cassava, beans and coconuts. They also keep a variety of domestic animals (cattle, goats, sheep and chicken). The town dwellers are involved in commercial activities, the hotel industry and local administrative jobs. Various forms of fuel are utilized by this community and include firewood, charcoal, gas or paraffin, their access to water being either open/closed wells or piped.

4.2 Study Design

4.2.1 Definitions

Acute respiratory infections (ARI) were classified as URTIs or LRTIs following modified World Health Organization (WHO) definitions for pneumonia (WHO, 2000; Table 4.1). An episode of LRTI was defined as the presence of cough or difficulty in breathing in addition to one or more of the following symptoms: chest in-drawing or increased respiratory rate. Children presenting with cough, nasal congestation/secretion or difficulty in breathing but no signs suggestive of LRTI were considered as having an URTI (Table 4.1). These definitions were mutually exclusive.

Table 4.1. Pneumonia classification at KDH for children aged less than 2 months (young infant) to 5 years (child) - adapted from the WHO guidelines (WHO, 2000). Acute cough or difficultly in breathing are prerequisite signs

Syndrome	Characteristic (Modified WHO)	Definition
Very severe pneumonia	(i) Prostrate (or inability to feed) (ii) Unconscious (iii) Oxygen saturation < 90% if accompanied by clinical LRTI diagnosis	Severe LRTI
Severe pneumonia	(i) Chest indrawing AND no sign of very severe pneumonia	Severe LRTI
Pneumonia	(i) Fast breathing for age* AND no signs of very severe or severe pneumonia	LRTI
No pneumonia	None of the above signs	URTI

*Fast breathing is present if the rate is ≥ 60 in those aged < 2 months, ≥ 50 if aged 2-11 months and ≥ 40 if 11 months or older.

4.2.2 Recruitment of cohort

About 300 children each were recruited in 2 phases to a birth cohort in Kilifi District. They were recruited between February – May 2002, and December 2002 – May 2003, respectively, were intensively monitored for ARIs as shown in the schematic below (Figure 4.2). Infants were recruited at birth, in the maternity ward, or within 2 weeks of birth, at the maternal child health clinic (MCHC) at KDH. A child was eligible if, in relation to KDH, the road access to their home was good, the travel cost (single) was < 50 Kenya shillings (US $0.7) and the journey time was <1 hour. Active household surveillance for ARI was weekly during RSV epidemics and monthly otherwise. Passive surveillance was carried out principally through parental referral to outpatient (OP) research clinic (Monday- Friday, 8 A.M- 5 P.M.) at KDH (Nokes et al., 2004). Passive referral was encouraged for a child with any ARI symptoms (or worse).

At each contact, a nasal washing (NW) was collected (Figure 4.3) if the child had ≥ 1 signs of acute cough, difficulty in breathing or if nasal congestion/discharge was observed or elicited by the history from the preceding week. A blood film for malaria diagnosis was collected if the child had a history of fever in previous week or an axillary temperature ≥ 37.5°C on the day of visit, with appropriate treatment being provided if found positive. Infant vaccination records were also checked, with advice to attend MCHC for any vaccination overdue. During an active visit, the child would be referred to the research clinic if she/he had a respiratory rate of ≥ 50 breaths/min if an infant, or ≥ 40 breaths/min if one year or over, and accompanied by cough or difficulty in breathing on the day of the visit. The number of complete weeks of absence from the district since the previous active visit was also recorded (Appendix II). Infants attending the OP research clinic underwent medical review by a clinical officer using a

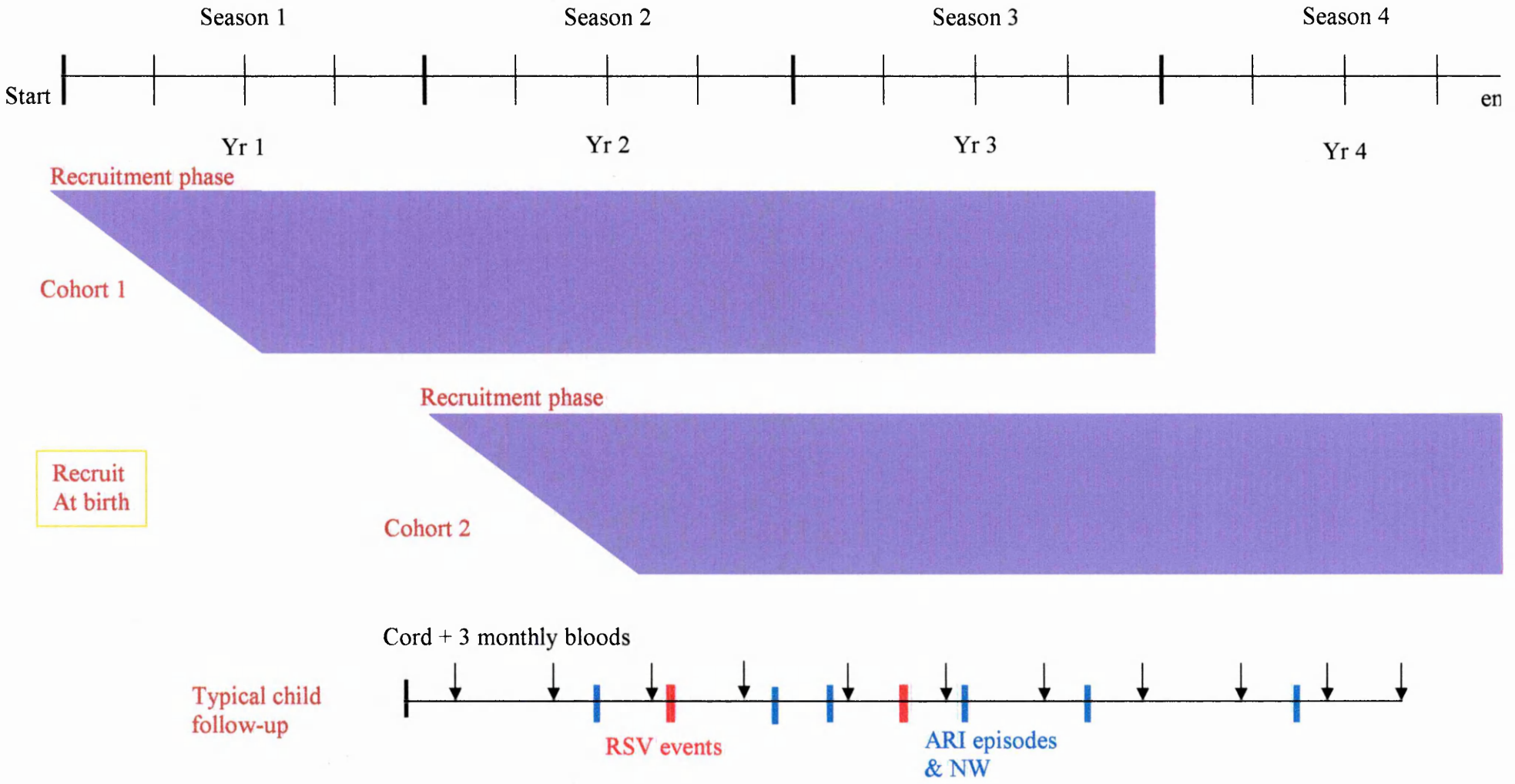


Figure 4.2. Study Design. Approximately 300 children were recruited at birth in each cohort, and each followed for a period of 3 years. Children in the birth cohort were recruited in 2 phases, 7 months apart. Cord bloods, together with sera every 3 months (as indicated by black arrows) were collected, as well, NW were collected upon each ARI episode (blue markers) and tested for RSV by IFAT (unless a confirmed RSV episode had arisen in preceding 14 days). If positive (red marker), paired acute & convalescent phase sera were subsequently collected. (IFAT, indirect immunofluorescence antigen test).

standardized pro forma method (Appendix III) to ascertain the severity of ARI. Transport costs were reimbursed and definitive medicines provided without charge. RSV-positive children were contacted as soon as possible following diagnosis, and the parents were requested to refer the child to the research clinic if his/her condition did not improve or deteriorated.

Figure 4.3. Collection of NW from child and infant respectively using a nasal wash bulb (NWB).

Additional passive surveillance of cohort infants was done through admissions to KDH (the majority of children could be linked to the admission records by their Epi-DSS number ascertained at the point of admission). NW were collected if the child presented with either severe or very severe pneumonia (Table 4.1) or had an admission diagnosis of LRTI. The baseline symptoms for sample collection in the field or at the OP clinic were runny nose/congestion, difficulty in breathing or cough (either on the day of visit or presentation, or with a history over the preceding 7 days).

Venous blood samples which included cord blood (when possible from maternity ward recruits) and repeated blood samples taken at approximately 3 monthly intervals were

collected from each child (Figure 4.2). If a child experienced a positive RSV event identified using IFAT, an acute phase blood sample was collected, and 1 month later, a convalescent sample. With regards to in- or out-patients, appointments were arranged for their return to the OP clinic, if the mothers failed to turn up on the appointed day however, their child (or children) was followed up at home.

4.3 General Characteristics of the Cohort

A total of 33,894 visits (passive, active or in-patient) were made and of these, approximately 78% were active whilst 21% were passive, with each child being visited between 1 and 104 times during the follow up period. Families ranged in size from 3-32 members of which the total number of children varied from 1-18 children. The average family comprised 4 children. Ninety-one percent of the births in this cohort were single births and infants were breast fed for 3-27 months. Thirty percent of these children were still being breast fed at the end of the follow-up period. Most children at birth were between 2.7- 3.4 kg in weight (Appendix I).

4.4 Ethical Considerations

The research project was one of several studies nested within a birth cohort study of RSV in Kilifi District, which was reviewed and passed as ethically acceptable by the Kenya Medical Research Institute/National Ethic Review Committee Kenya and Coventry Research Ethics Committee, UK. Informed consent (Appendix IV) for participation in the study was obtained from each infant's mother (Nokes et al., 2004).

4.5 Methods

The suppliers of all the reagents and consumables used in these studies are listed in Appendix V.

4.5.1 Sample collection

4.5.1.1 Nasal washings (NW) and RSV diagnosis

NW were collected by the method of Hall & Douglas (Hall & Douglas, 1975), that utilized a NWB (Figure 4.3) and 5 mL of normal saline for infants <6 months old, 7 mL for those 7-11 months old, and 10ml if older than 11 months. Samples collected in the household were stored in a cool box and transported to the microbiology laboratory at the end of the day; those collected in the OP clinic were delivered within 1 hour. Samples were stored at 4°C until screening for RSV antigen using light diagnostics™ RSV direct immunofluorescence assay (DFA) as per the manufacturer's (Chemicon International Inc.) protocol. This usually occurred within 24 hours and invariably within 3 days. Briefly, mucus was broken up by pipetting up and down several times and then slides prepared from 200 µL of specimen by use of a cytology centrifuge (67 X g, 10 min). Following this, slides were air-dried, fixed in chilled acetone (2-8°C) for 10 min and then a drop of RSV/FITC (fluorescein isothiocyanate) Ab placed on re-dried slides. Slides were placed at 37°C in a humid chamber for 30 min, air-dried before mounting with mounting fluid, whereupon they were examined under a fluorescent microscope (X 20- 40 objective). This assay is an immunoflurorescence antigen test (IFAT) and cells in positive specimens fluoresced apple green, while uninfected cells, stained dull red (counter stain).

4.5.1.2 Blood collection

The field worker used a butterfly needle (B-23G) and syringe (5 mL) to collect venous blood in the home of each participating individual. If any failure occurred, a second attempt would be made whilst out in the field, and in the OP clinic, one of the attending medical personnel would try a further time, before indicating on the form that no blood was drawn. For individuals attending the OP clinic, venous blood was withdrawn also using a butterfly needle (B-23G). Blood (2 mL) was placed in a bijou bottle, and transported to the laboratory. Those collected in the OP clinic were delivered within an hour of collection, whilst those in the field were stored in a cool box and transported upon return at the end of the day. In the microbiology laboratory, samples were left to clot at 4°C, then centrifuged (1400 X g for 5 min) and serum collected by use of a plastic Pasteur pipette, into an apex (2 mL) tube. Samples were stored at –70°C until further analysis.

Both thick and thin blood smears were prepared from a finger prick. Slides were transported back to the laboratory for processing as earlier described (Warhurst & Williams, 1996). Briefly, slides were fixed by immersion into methanol (30- 60 sec), air-dried and then stained with 10% Giemsa in phosphate buffered water (20-30 min), rinsed (tap water) and then left to dry vertically before examining for malaria parasites (X100 magnification) by personnel in the Haematology laboratory.

Serum from 6 adults was collected for later use as reference and controls in the ELISA (described later). From each of 6 adults, 5 mL of venous blood was withdrawn using a sterile needle and syringe. Samples were immediately taken to the Microbiology laboratory, left to clot at 4°C, centrifuged (3000g for 5 min) and sera from 5 of these 6 sera pooled by using a

plastic Pasteur pipette into a 15 mL falcon tube. Pooled sera, as well as the remaining serum, were kept as 250 µL aliquots at –20°C until further use.

4.5.2 Cell and virus culture

4.5.2.1 Culture media

Culture media was made up using minimal essential medium with Earles salts and L-glutamine (MEM) which was supplemented with 1M HEPES buffer, non-essential amino acids (100X), penicillin/streptomycin (10,000U/mL penicillin and 10 mg/mL streptomycin diluted 1:100) and 5% fetal calf serum (FCS). Maintenance MEM was made as above, except 2% FCS was used.

4.5.2.2 Cell culture

HEp-2 cells were retrieved from liquid nitrogen cryopreservation and placed gently into 5-7 mL pre-warmed (37°C) supplemented MEM (CMEM) in 25 cm^2 tissue culture flasks (T-25 cm^2). All flasks were incubated at 37°C in a 5% CO_2 enriched atmosphere. Cells were examined microscopically using phase contrast, after 24 hours. Culture medium was initially changed 24 hours following retrieval, and then every second day thereafter, sub-culturing as necessary into tissue culture flasks of the same size or scaling up into T-75 cm^2 or T-150 cm^2.

Sub-culturing of cells was carried out as follows. Spent medium was removed from confluent cell monolayers, which were washed twice using a volume equivalent to half the volume of culture medium with phosphate buffer saline (PBS) without Ca^{2+}/Mg^{2+}. Cells were then incubated with 1 mL trypsin/EDTA per 25 cm^2 of surface area until they began to detach; this

was microscopically confirmed. The side of the flask was gently tapped to dislodge the remaining attached cells, then the reaction quenched by adding the appropriate volume of CMEM. The required number of cells was transferred into new flasks containing pre-warmed CMEM. All flasks were incubated as before.

4.5.2.3 Cryopreservation of HEp-2 cell lines

Flasks (T-150 cm^2) were viewed microscopically to confirm the degree of cell density and confirm the absence of bacterial and fungal contaminants. Cell viability was in excess of 90% in order to achieve a good recovery after freezing. Adherent and semi-adherent cells were brought into suspension using typsin/EDTA and cells re-suspended in fresh CMEM as above. Cells were centrifuged (157 X g, 10 min) and supernatant carefully decanted, and then the pellet gently re-suspended at a concentration of 2-4 X 10^6 cells per ml in freezing medium (10% dimethyl sulphoxide, 50% FCS in CMEM). Aliquots of 1 mL of cells were placed in appropriately labeled cryoprotective ampoules. Ampoules were placed inside a passive freezer (Nalgene Mr Frosty) and placed at –80°C overnight; these were then transferred to liquid nitrogen.

4.5.2.4 Virus stock preparation

A semi-confluent (50-70%) T-25 cm^2 flask of HEp-2 cells was used and spent medium decanted. One mL of 1:1 ratio of maintenance MEM and RSV type A2 strain (ATCC VR1540) was inoculated onto these cells. Flasks were then incubated at 33°C in a 5% enriched CO_2 atmosphere, for 2-3 hours, with gentle swirling every 15 min to allow for virus attachment. Maintenance MEM was added to make up to the appropriate volume. The flasks

were re-incubated and checked on a daily basis for signs of syncytia formation and cytopathic effects (CPE) by the majority of cells, which were also characterized by a change in pH in the medium. Uninfected flasks were set up in parallel as controls. When 50-70% CPE occurred, sterile glass beads were utilized to gently dislodge the disrupted monolayer and the resulting cell suspension aliquoted (1 mL) into ampoules and stored at -70°C until further use.

4.5.2.5 Viral antigen (lysate) preparation

HEp-2 cells in T-150 cm^2 flasks were infected with RSV A2 strain as described above at 33°C. When extensive CPE was observed (Appendix VI), usually 3-5 days post-infection, mock and infected cell lysates were prepared by scraping cells off into the medium. Cells were then pelleted by centrifugation (352 X g for 10 min) and resuspended in 10 mL PBS. A further centrifugation step was carried out (352 X g for 10 min) before resuspending pellet in 0.5% Nonidet-P40 (NP40) in 10 mL water. The suspension was thoroughly mixed by whirli-mixing and then debris pelleted (352 X g for 10 min). The supernatant was aliquoted and stored at -70°C until required.

4.5.2.6 Enzyme linked immunosorbent assay (ELISA) procedure

The method used was adapted from Wilson et al (2000) used for the detection of RSV specific IgG in oral fluid. Ninety-six-well plates (Immulon-2 HB) were coated overnight with 50 µL of appropriate dilutions of either RSV-A2-infected or mock-infected cell lysates (chapter 5) prepared as described above, and allowed to dry within wells. These constituted the test and control wells, respectively. Plates were then fixed at room temperature for 10 min using 80% acetone, drained and allowed to dry, then stored at –20°C until use.

The ELISA procedure was carried out as follows. The optimal concentration of reagents and sera was determined by checkerboard titration (chapter 5). Plates were blocked with 200 µL/well of 5% dried skimmed milk powder in PBS-Tween (0.05% Tween, PBS-T) at 37°C for 60 min. 100 µL of serum, appropriately diluted in blocking buffer, were carried out, then added in duplicate to both RSV-A2 and mock lysates for 90 min. Plates were washed four times with PBS-T, followed by incubation with 100 µL horseradish peroxidase-conjugated rabbit antihuman IgG (1:1000 dilution in 5% dried skimmed milk powder in PBS-T) at 37°C for 60 min. Plates were washed again four times in PBS-T. 100 µL of freshly prepared O-phenylenediamine (30 mg tablet dissolved in 30 mL PBS with 30 µL H_2O_2) substrate was added for 10 min in the dark. The reaction was quenched with 50 µL (2.5 mol/L) sulphuric acid and absorbance (OD) read at a wavelength of 492 nm using an ELISA plate reader. Further details of the optimization of assay are presented later in the following chapter. An OD of >0.2 was considered positive following subtraction of corresponding mock-infected cell lysate reading (Wilson et al., 2000).

4.5.2.7 Modified lysate preparation/ELISA procedure

The original lysate preparation and ELISA assays (sections 4.5.2.5 & 4.5.2.6) resulted in inconsistencies (see chapter 5 for further details) and therefore both assays were modified further (for further details see Appendix VIII). Briefly, lysate preparation was carried out as previously described above (4.5.2.5), however, prior to storage at –70°C, supernatant (10 mL) was sonicated (70% amplitude, 3X 1 min cycles with 1s pulse and 1s pause) and resulting lysate pooled then vortexed thoroughly. Lysate was then stored.

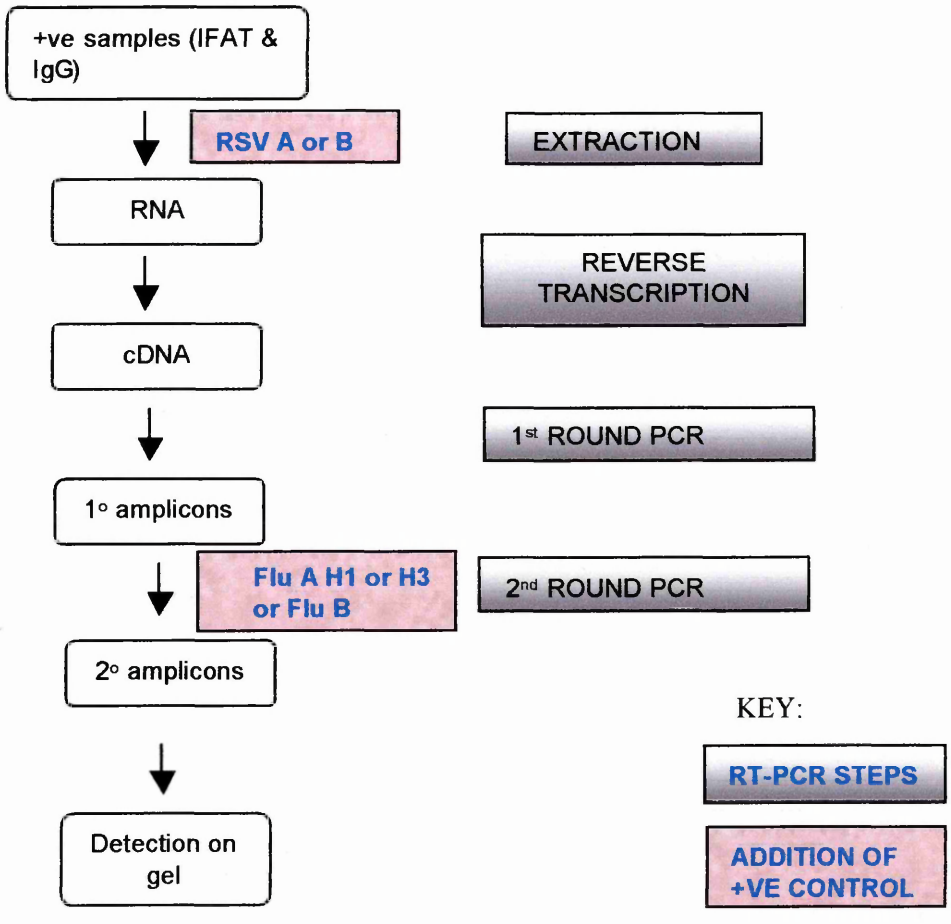
Ninety-six well plates (Nunc-Immuno™ MaxiSorp™) were used. 25 µL of appropriately diluted lysate in PBS coating buffer was placed in each well and allowed to adsorb over night (37°C) in a rotating incubator then coating buffer flicked off before proceeding with ELISA as previously described above (4.5.2.6) with one modification. Blocking buffer comprised 5% skimmed milk powder in PBS only.

4.5.3 Molecular assays

4.5.3.1 Clinical specimens

RSV IFAT positive NW specimens collected by both active and passive surveillance as earlier described, as well as those samples that were shown to be IFAT negative but positive by ELISA for RSV-specific IgG, (see chapter 6, section 6.3) were tested. The positive controls included HEp-2 cells infected with either RSV A or B which were used in all PCR runs, and the first round cloned PCR products (kindly supplied by Dr Paul Scott, Warwick University) for the remaining virus strains (Influenza A H1 and H3 strains, and Influenza B) was incorporated into the assay (Figure 4.4). First round products (Influenza controls) were included during the secondary round. This thus allowed for discrimination between all 5 viral amplicons. Negative controls included template-free reaction tubes.

Figure 4.4. Flow chart illustrating addition of positive controls during the RT-PCR assay



4.5.3.2 Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis by reverse transcription

Ribonucleic acid (RNA) from NW was extracted using the QIAamp Viral RNA Mini kit following the manufacture's instructions with the following modification: carrier RNA was omitted. (All buffers were supplied by manufacturer). Briefly, AVL buffer (560 µL) was added to NW (140 µL), vortexed and then incubated at room temperature (10 min). Absolute ethanol (560 µL) was added, the sample mixed by vortexing and then carefully transferred to QIAamp spin column (in a 2 mL collection tube). A centrifugation (6000 X g, 1 min) step followed. After each centrifugation step, the spin column was placed in a clean collection tube. The remainder of the sample was added, and centrifugation was repeated. Buffer AW1

(500 µL), was added, and the tube then centrifuged. This was followed by addition of buffer AW2 (500 µL), centrifugation at full speed (20,000 X g, 3 min) and finally, buffer AVE (60 µL) added. The column was incubated at room temperature (1 min) and a final spin carried out (6000 X g, 1 min). The resultant eluted RNA was stored at –20°C or used immediately to synthesise complementary deoxyribonucleic acid (cDNA). Following extraction, cDNA synthesis was carried out using reagents from the Qiagen Omniscript Reverse Transcriptase kit. 20 µl of RNA was added to a reaction mix (20 µl) containing 10× RT (reverse transcriptase) buffer, 5 mM of each deoxynucleoside triphosphate, Omniscript reverse transcriptase (200 units) and 250 ng/µl random hexamers [pd(N)$_6$]. The reaction mixture was incubated at 37°C for 60 min. Reverse-transcription reactions were stored on ice if proceeding directly with PCR, or for long-term storage at –20°C.

4.5.3.3 Primers used

Previously described primers (Cane & Pringle, 1991, Stockton et al., 1998) were utilized. Each primer was 20 nucleotides long with a base composition that ranged from 50-60% in GC content. Primers were synthesized by Alta Biosciences, Birmingham, and these are listed in Appendix VII.

4.5.3.4 Multiplex polymerase chain reaction (mPCR)

Following cDNA synthesis, the method of multiplex polymerase chain reaction (mPCR) as described by Stockton et al. (1998), was used. First round primers (Appendix VII) at 5 µM were used to carry out the primary amplification step and 2.5 µM of second round primers (Appendix VII) for the secondary amplification. For the primary PCR round, 20 µL of cDNA

was added to 80 μL of a reaction mix containing Taq PCR Master Mix and 25 mM $MgCl_2$. Amplification with a DNA Engine thermocycler consisted of 1 cycle at 94°C for 2 min; 35 cycles of 94°C for 1 min; 50°C for 1 min and 72°C for 1 min; 1 extension cycle at 72°C for 5 min and then cooled. Two microlitres of the primary product was then transferred to 48 μl of secondary amplification mixture as above. The samples were then incubated for 1 cycle at 94°C for 2 min; 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and 1 extension cycle at 72°C for 5 min and then cooled. Resulting amplicons were visualized by gel electrophoresis.

4.5.3.5 Gel electrophoresis

Each amplicon (8 μL) was mixed with loading buffer (2 μL), in addition, 5 μL 100bp DNA ladder (5+ Units) in 2 μL loading buffer were loaded onto a 1% agarose gel in 0.5x TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) stained with ethidium bromide. Electrophoretic separation was performed for 30 min at 130 mA. Following electrophoresis, the remaining aliquot of products was stored at –70°C until further use.

4.5.3.6 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit following the manufacture's instructions, prior to undergoing further analysis. Briefly, buffer PB (5 volumes) was added to PCR sample (1 volume) and mixed thoroughly. The sample was added to a QIAquick spin column that was previously placed in a collection tube and centrifuged (≥ 10, 000 X g, 30-60 s). Flow-through was discarded, the spin column placed in a clean

collection tube and buffer PE (0.75 mL) added. The sample was centrifuged (≥ 10, 000 X g, 30-60 s) again, flow-through discarded and spin column replaced in the same tube for a another centrifugation step (≥ 10, 000 X g, 1 min). The spin column was then placed in a clean 1.5 mL microcentrifuge tube and 50 µL of buffer EB (10mM Tris-Cl, pH 8.5) or water added to the spin column (1 min) and then centrifuged (≥ 10, 000 X g, 30-60 seconds). The purified product was stored at -20°C until further use.

4.5.3.7 N gene PCR

This was carried out as a nested PCR using the purified positive first round products synthesized from the mPCR protocol above, and N gene primer pair at 10 pmol/µl as earlier described (Cane & Pringle, 1991). One microlitre of first round mPCR product was added to 49 µl of a reaction mix containing Taq PCR Master Mix, and then incubated for 1 cycle at 95°C for 2 min; followed by 35 cycles of 95°C for 45 s, 54°C for 45 s and 72°C for 1 min; and finally 1 extension cycle at 72°C for 5 min and then cooled. Amplicons were visualized following electrophoresis on 1% agarose gel as above. All products were stored at –70°C until further use.

4.5.3.8 N gene restriction fragment length polymorphism

N gene typing by restriction fragment length polymorphism (RFLP) was carried out by digesting N gene PCR products at 37°C for 1 hour (Cane & Pringle, 1991) with Hind III, Pst I, Bgl II, Hae III and Rsa I. Amplicons were visualized by ethidium bromide staining following electrophoresis as described above but using a 2% agarose gel instead. The resulting banding patterns were then used to determine the N gene pattern (NP, see table 6.2).

4.5.4 Methods for statistical analysis

All data analysis was carried out using Stata 9.0™ and will be described in detail later, refer to sections 7.5 and 8.4.

CHAPTER FIVE

RSV-specific Enzyme Linked Immunosorbent Assay (ELISA): Assay Development

5.1 Introduction

Previously, complement-fixation (CF) and hemagglutination inhibition (HAI) tests were used as the basis for serological diagnosis of viral infections (Julkunen, 1984, Yolken, 1980). The immunological response to RSV infection was measured by the detection of a rise in CF Abs in convalescent phase relative to acute phase serum from infected individuals. However, serological investigation of RSV can be unreliable when performed by CF on infants (Steinhoff et al., 1980) due to its reduced sensitivity: titres measured by CF have been shown to be lower in comparison to ELISA (Hornsleth et al., 1984, Richardson et al., 1978), with some authors describing Ab titre levels of up to 100-fold lower (Richardson et al., 1978) than those obtained by ELISA.

In comparison with other serological techniques such as the plaque reduction assay, it has been stated that the ELISA technique detects a serological response to RSV in approximately 50% of infants under 6 months of age infected with the virus, *i.e.* those most vulnerable to serious disease (Richardson et al., 1978, Meurman et al., 1984). The plaque reduction assay measures only those serum Abs which neutralize virus and therefore render it incapable of initiating a plaque *i.e.* Abs to one or more surface antigens, whereas the ELISA measures both neutralizing and many other Abs directed against internal epitopes and other epitopes that bind Abs without virus neutralization. Non-neutralizing antigens are particularly abundant in RSV-infected cells (Richardson et al., 1978). Abs detected in ELISA should be characterized as

binding rather than neutralizing and caution must be exercised in the interpretation of ELISA results, as the Abs measured may not be protective. This assay therefore, should not act as a surrogate for protective immunity. Various applications of ELISA for the measurement of RSV serology have been carried out by using various antigens which include purified antigens (Nandapalan et al., 1984), cell lysate antigens (Steinhoff et al., 1980) or crude extracts from infected cells (Richardson et al., 1978).

Over the past 20 years, enzyme immunoassay methods have been developed for the diagnosis of viral infections of members of the *Paramyxoviridae* family such as parainfluenza virus, measles virus (Julkunen, 1984, Kacica et al., 1995, Kurstak et al., 1986, Yolken, 1980) and RSV (Steinhoff et al., 1980). ELISAs have also become an important alternative to cell-culture techniques (Kurstak et al., 1986) allowing for diagnosis of infection within hours rather than the traditional 2 or 3 weeks. The ELISA is both reproducible and an objective test that can be performed on small amounts of serum (Steinhoff et al., 1980). The versatile nature of the ELISA, the ability to adapt this assay to microplate format, to detect isotype-specific or isotype subclass-specific responses, its relatively cheap cost, the relative ease of performance, the rapidity with which test results are made available, in addition to its sensitivity, led us to adopt the assay for analysis of serum samples collected from the birth cohort which was followed for 3 calender years.

An indirect ELISA method using a crude extract from RSV infected cells and mock infected cells, coated onto 96 well plates was used in this project. This method was attractive as it did not require any antigen purification or cellular treatment. For all Ab titrations, all specimens (control, standard or reference and test sera diluted appropriately) were tested in parallel

against both RSV and control (mock) antigen-coated wells. The primary objective was primarily to identify infections, *i.e.* determine RSV-specific Ab responses between paired acute and convalescent samples (for which the ELISA is well adapted) in the presence or absence of matAbs. Secondarily, the ELISA was to be used to detect matAb decay, and to identify protective levels of Ab. Moreover, it was envisaged that the assay would also detect missed infections, in addition to confirming RSV IFAT (clinically identified) positive results. Additionally, the assay being developed had to be reproducible, accounting for variation within and between plates and from one batch to the next.

The setting of appropriate assay cut-off values is of importance in the development procedure as a means of evaluating assay performance. A further consideration is the availability of well characterized positive, and particularly negative controls, that have usually been assessed using the gold standard (Kurstak, 1985). The mean result of a population of negative samples confirmed from the gold standard assay, plus either 2 or 3 standard deviations is a commonly used method for setting the cut-off value. The appropriate cut-off values will thus be evaluated for our assay, following optimization of assay conditions.

5.2 Objectives

The main objective of this chapter was the development of a simple, sensitive and robust ELISA-based Ab detection method for RSV that is reproducible with minimal inter- and intra-plate variation.

Specifically the objectives included the following:

- Investigation of whether the optimized ELISA could measure the decay kinetics of RSV-specific matAb and the resulting dose response curves in the presence or absence of an infection.
- Identification of the dynamic range of the log linear phase, and hence assess whether this phase for each child is parallel; this therefore allowed for the determination of an optimal dilution to assay serum samples.
- Identification of both high and low saturation zones of dose response curves in which the assay is insensitive to changes in Ab concentration and hence determine high and low assay cut-off titres respectively.
- Determination of whether the assay parameters and chosen dilutions could resolve serologically a recent infection event in the presence or absence of matAbs.

5.3 Results

5.3.1 Optimization of ELISA Procedure

Initially, optimization of the ELISA parameters (dilution of capture antigen, conjugate dilution and so on) was undertaken before going on to further investigate the sensitivity of the assay.

A variety of stages in the indirect ELISA can be varied to obtain the desired characteristics of assay performance and these have been previously described (Hendry & McIntosh, 1982, Richardson et al., 1978, Wilson et al., 2000). What follows is an account of a series of experiments performed to obtain assay performance suitable to move to large-scale screening. A systematic approach was adopted to modify only one or two stages or components of the assay at any one time.

5.3.2 Determination of dilution of capture antigen

5.3.2.1 Method

Previously harvested lysate was used to coat 96 well plates, as described in Materials and Methods (Section 4.5.2.5). A 96 well plate was divided into half lengthwise and widthwise. The first 6 columns were coated with RSV A2-infected lysate, whilst the next 6 columns with mock infected control lysate. Wells in rows A-C were coated in triplicate with doubling dilutions of appropriate lysate, starting with a starting dilution of 1:2 to 1:64; this was repeated in rows E-G. Rows D and H were not coated and acted as control for the Ab (Figure 5.1). Initially, two monoclonal Abs against the G protein, O21/1G and O21/2G [Kindly donated to Dr. P Cane by Dr. J.A. Melero, (Madrid, Spain)] were used at a dilution of 1:500 as primary Ab in rows A-D and E-G respectively. Subsequently, adult sera at a dilution of 1:100 were used once it was confirmed that harvested lysate showed specific reaction to RSV Abs. The rest of the ELISA procedure is as previously described before (Section 4.5.2.6).

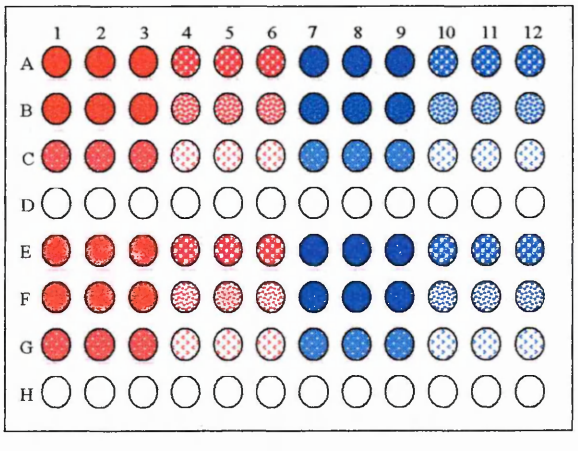


Figure 5.1. Schematic of layout of ELISA plate. Red and blue circles depict RSV infected and mock infected lysates respectively. Lysate dilutions in rows A-C are depicted as follows, ○ 1:2; ○ 1:4; ○ 1:8; ○ 1:16; ○ 1:32; ○ 1:64. This was repeated in E-G.

5.3.2.2 Results

Figure 5.2 illustrates the signal: noise ratios observed for each dilution of capture antigen and respective mock lysates. The best lysate dilution that gave a good positive signal with the infected lysate but not uninfected lysate was determined for each lysate preparation and shown to occur at dilutions of between 1:8 to 1:64.

5.3.2.3 Interpretation

Using lysates at concentrations higher than 1:8 was not feasible in terms of the logistics of lysate production. Suitable dilutions following 6 experiments fell between 1:32 to 1:64; therefore the concentration used was 1:64.

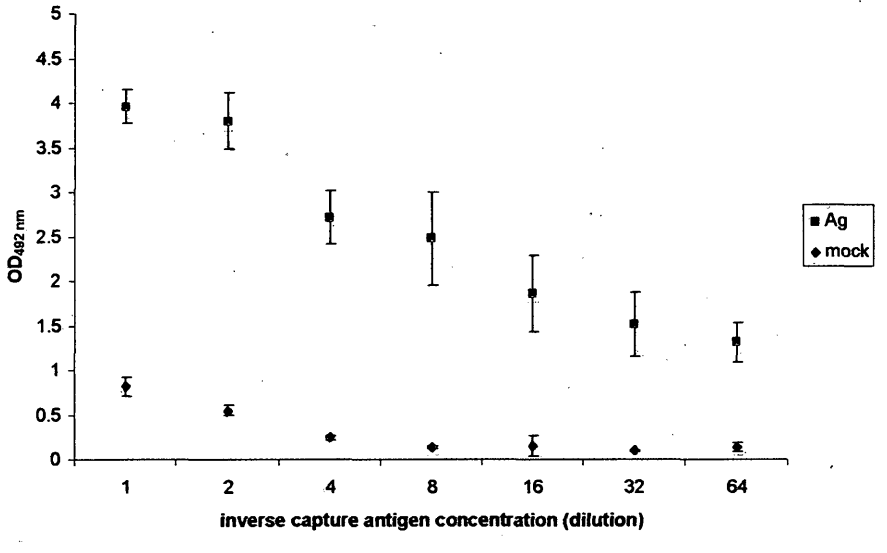


Figure 5.2. The signal: noise ratio for different capture antigen dilutions (serum tested 1:100). Dilutions carried out in triplicate; Ag and mock are the virus infected and mock infected lysates, respectively. Bars indicate standard deviation.

5.3.3 Determination of dilution of rabbit anti-human IgG conjugate

5.3.3.1 Method

Three adult sera were titrated in triplicate against antigen in a checkerboard assay. Four-fold serial dilution starting at 1:50 to 1:12,800 of serum was titrated against conjugate at the following dilutions: 1:500, 1:1000, 1:2000 and 1:4000.

5.3.3.2 Results

OD readings with conjugate at 1:4000 for all test sera were observed to be too low and this dilution was subsequently omitted in repeat analysis; conjugate at 1:1000 gave higher ODs at a dilution of 1:50 for all test sera. With test sera at a dilution of 1:200 and conjugate at 1:1000, this gave higher or similar results to the other dilutions.

5.3.3.3 Interpretation

Therefore, a working dilution of 1:1000 of rabbit anti-human conjugate was adopted for subsequent assays.

5.3.4 Reproducibility of ELISA

The ability of the assay to produce reproducible ELISA OD values over time was tested using sera from healthy Kenyan adults, and this was repeatedly tested over one month.

5.3.4.1 Method

Sera from 3 healthy Kenyan adults were tested using the assay 15 times, initially in duplicate and then in triplicate, over a period of 1 month. A two-fold serial dilution, starting at 1:200 to 1:6400, was carried out on all test sera. The starting serum dilution was 1:200 as this was earlier observed to give good signal, during the optimization process for the conjugate.

5.3.4.2 Results

The OD_{492} decreased with increasing serum dilution (see Figure 5.2). The dynamic range observed was wide, with OD_{492} values ranging from 0.02-1.9. At the outset OD_{492} varied from 0.2-0.5 between replicate readings, with time this improved to within a range of 0.06-0.16 with background ODs remaining relatively low. However, there appeared to be a systematic decrease in OD for triplicate readings over time as illustrated in Figure 5.3. Additionally, the coefficient of variance within plates was seen to be 15-17% and similarly high between plates.

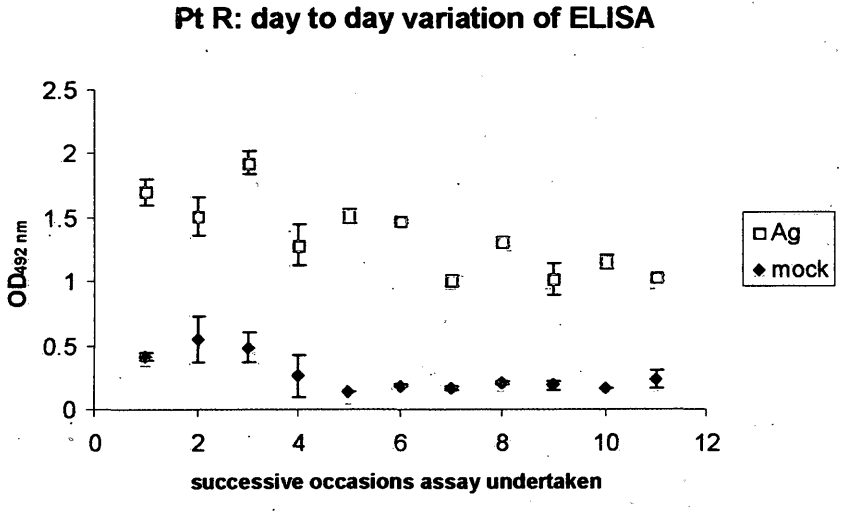


Figure 5.3. The day-to-day variation over a period of 1 month for Pt R (serum tested at 1:200 dilution) showing dynamic range of observed ODs and low background values. Ag and mock are the virus infected and mock infected lysates respectively. Bars indicate standard deviation.

5.3.4.3 Interpretation

Due to the variation noted over successive occasions, it was decided that (a) all samples for one child should be screened on the same day and, (b) a set of standards should be used consistently to take into account this variation. With respect to the high coefficient of variance, new batches of antigen were produced and tested in parallel.

5.4 Sensitivity of ELISA: Validation of ELISA Method and Optimization of Serum Dilution

As the ELISA was shown to be reproducible on establishing the optimal dilutions of capture antigen and conjugate, the sensitivity of the assay was investigated. This was done by testing a subset of samples with different expected titres, inclusive of convalescent sera, which were anticipated to exhibit the highest titre, together with known negatives (sera collected during the inter-epidemic period where the likelihood of a child being infected was minimal therefore there would be no RSV-specific Ab detected) as a means of identifying the dynamic range, optimal dilution(s) to assay test sera and control, as well as assay cut-off values and the level of inherent assay variation expected. Thus, this would give an indication of the levels of saturation at high titres, in addition to what constituted too dilute a concentration with respect to samples with possibly "low" Ab titres, such as acute samples.

5.4.1 Dynamic range of dose response curves

5.4.1.1 Method

Initially, sera from 5 inpatient infants whose ages ranged from 0-2 months (it was expected that matAbs would be at maximal titres during this age range) were used. Two-fold dilutions ranging from 1:20 to 1:40,960 were carried out. Subsequently, sera of patients from the cohort, the target population, were examined over a wider age range (0-12 mos) in the validation process as this took into consideration sera of both high and low titres, to give a better assessment of the assay.

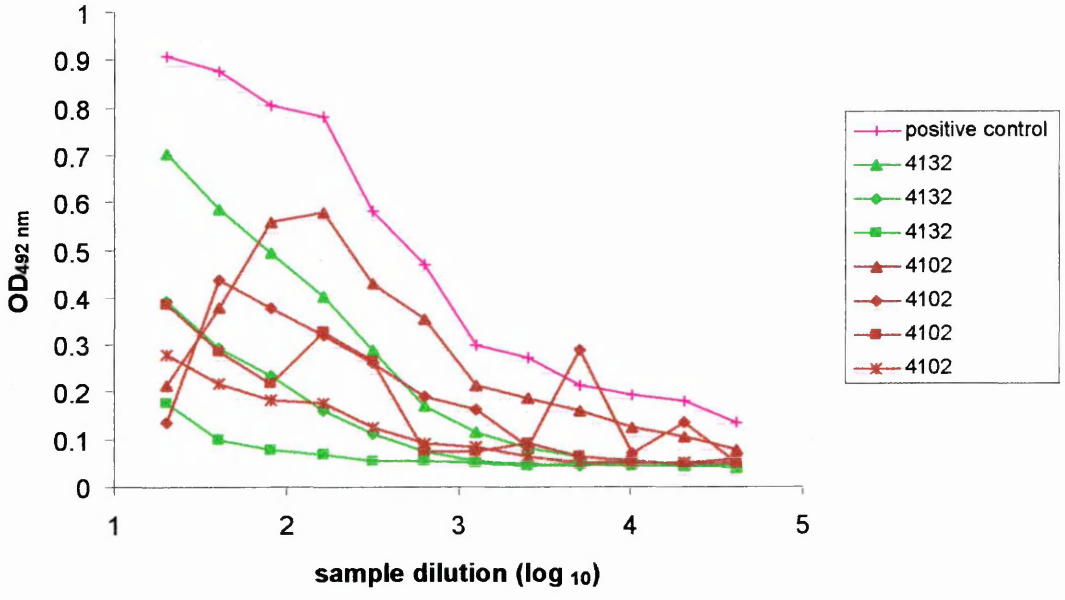
5.4.1.2 Results

It was noted that the dose response curves for most patients were approximately sigmoidal (Figure 5.4); occasionally, the prozone or (high-dose) hook effect was noted as for Pt 4102-0 mos (Figure 5.4A) and Pt 3735-6 mos (Figure 5.4B). Not all samples showed saturation at the highest concentration. All samples plateaued at the highest dilutions.

5.4.1.3 Interpretation

With increasing dilution, from 1:20 to 1:40,960, the typical dose-response curve for children tested was approximately sigmoidal. Furthermore, with increasing age it appeared that there was a decrease in specific Ab levels, most likely to be the decay in matAb. For most children tested, by the age of 12 months there was negligible Ab detected.

A



B

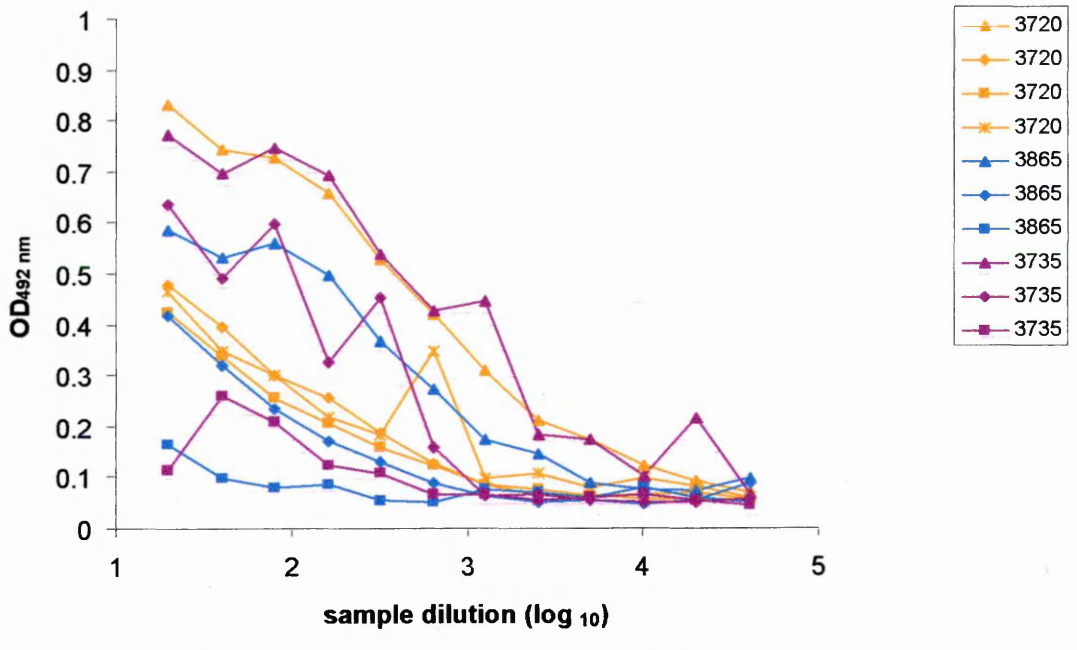


Figure 5.4. Adjusted (test-mock) average dose response curves of 3-monthly samples (0-9 months) double diluted (1:20-1:40,960) for a group of selected infants. Each patient is represented by a different colour and the symbols represent age groups: 0 months; 3 months; 6 months; 9 months of age. Each point represents average of duplicate OD readings.

5.4.2 Identification of the dynamic range of the log linear phase

5.4.2.1 Test for parallelism

An important problem with Ab dose-response curves is that differences in Ab concentration and affinity means that these curves are not always parallel (Balfour & Harford, 1990). Thus, at one dilution a given serum may appear to have the higher Ab level, but at a different dilution - where the curves crossed – the relative Ab level would appear lower (Kurstak, 1985). With this in mind, it was ascertained whether dose response curves were parallel or not.

5.4.2.1.1 Method

The indirect ELISA was carried out on 3-monthly samples from 8 patients over the range 1:20 to 1:40,960. Log linear portions of each curve were then plotted (Figure 5.5a) and following on from this, the means of gradients, with corresponding standard deviations calculated and plotted (Figure 5.5b)

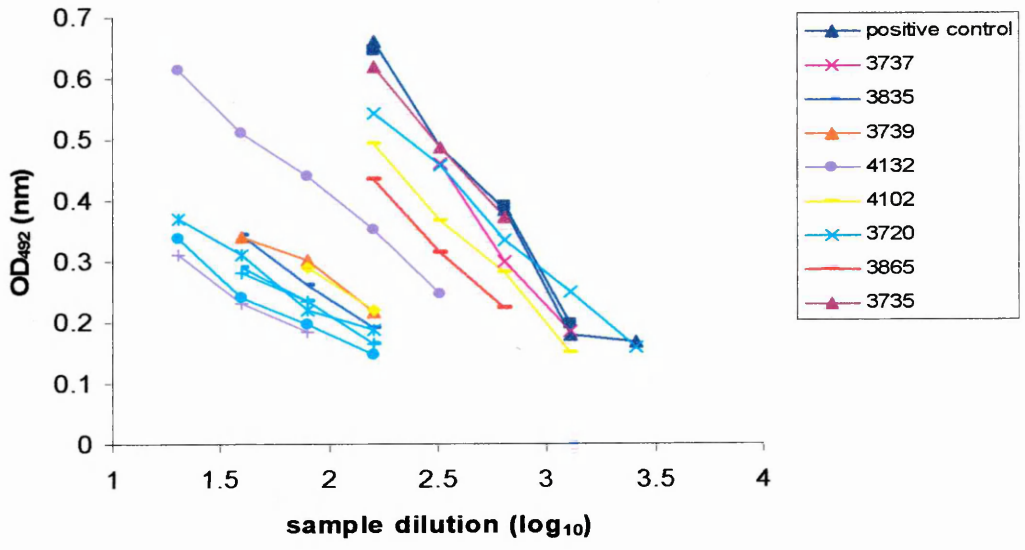
5.4.2.1.2 Results

There was no significant difference between gradients of test samples based on observations rather than statistical analysis as shown below (Figure 5.5b).

5.4.2.1.3 Interpretation

Hence, sample dose response curves were parallel and it is therefore possible to use 1 dilution in future assays.

A



B

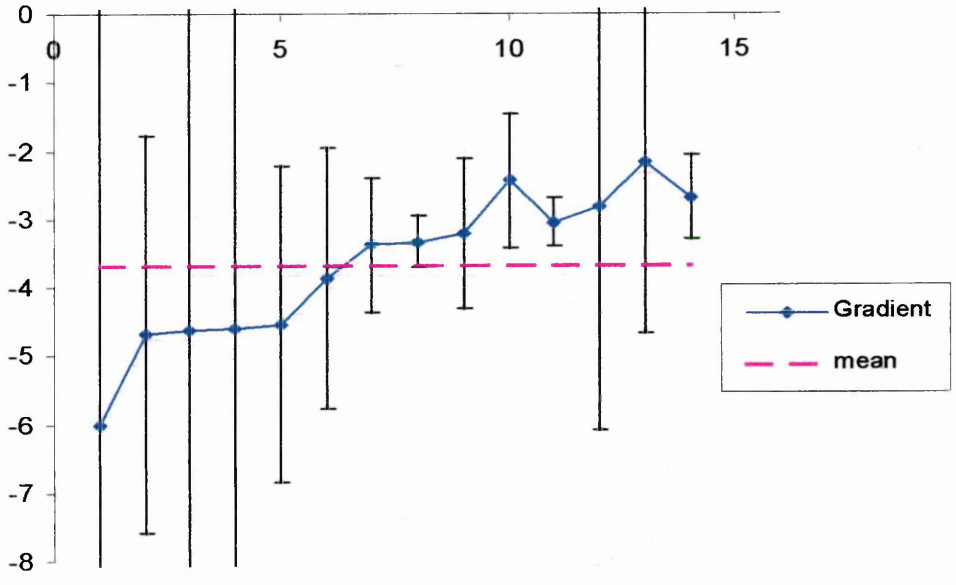


Figure 5.5. Panel A, Linear portions of 3-monthly samples serially diluted (1:20-1:40,960) for 8 infants (0-12 mos). Each point represents duplicate adjusted (test-mock) OD readings. Each patient is represented by one colour; different markers depict various 3 monthly samples. Panel B, plot of gradients for these 8 infants. Bars denote standard deviation.

5.4.3 Further characterization of log linear phase

Further analysis of the various dose response curves generated was carried out to ascertain the range of the log linear phase as age increased (0-12 months), as well as dilution (1:20 to 1:40,960). Initially only cord and 3-month samples were investigated. It was noted that the log linear phase of the sigmoid curves fell between 1:80 to 1:2560 for cord or 0-month samples, the lower saturation zone occurring below 1:80 dilution; whilst for 3-monthly samples this saturation occurred later, at dilutions below 1:640. As such, a dilution of 1:100 was chosen as the working dilution as this occurred in the straight part of the curve, in other words, the portion where the highest sensitivity could be measured. To confirm the validity of this chosen dilution as age varied, additional analysis was carried out on the log linear portions of the remaining age groups. Only plots for cord bloods, 3- and 6- month samples are depicted below, Figure 5.6a-d. Figure 5.6d is a plot for 1 patient as a typical example of the relationship of the linear portions of the 3 monthly samples; also shown is proposed working dilution of 1:100.

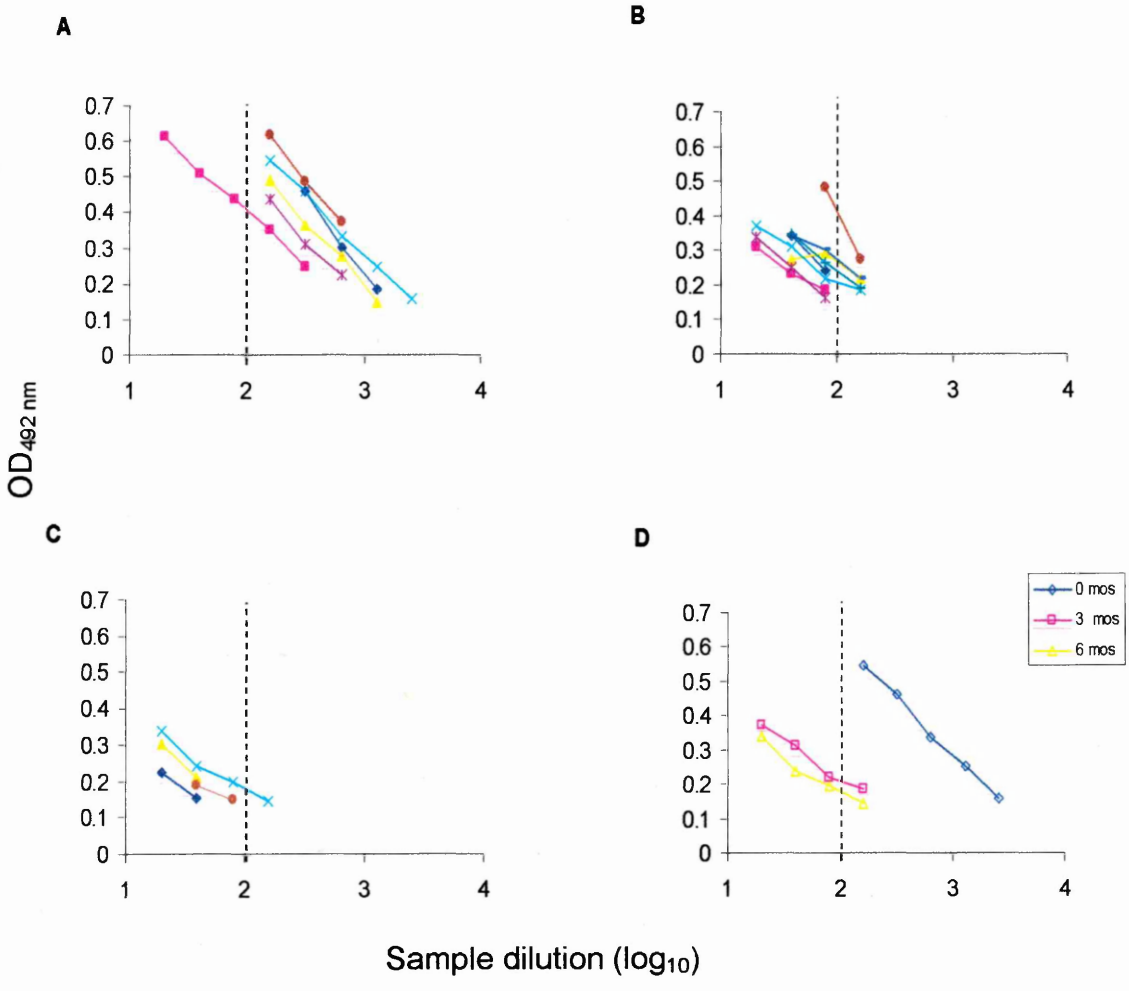


Figure 5.6. The linear portions of serially diluted (1:20-1:40,960) sera of selected patients. Panel A-C are cord, 3-monthly and 6-monthly sera respectively, panel D is a plot of 3 monthly samples of Pt 3720. Each point represents duplicate adjusted (test-mock) OD readings. In panel A-C the symbols represent the following patients: Pt 3737; Pt 4132; Pt 4102; Pt 3720; Pt 3865; Pt 3735. In panels A-D dashed line, - - - denotes 1:100 dilution (proposed working dilution).

5.5 Reference/Standard Serum and Controls

RSV IgG positive sera from healthy Kenyan adult donors were identified during the ELISA procedure and shown to have different levels of IgG to RSV. Pooled adult sera, constituting reference serum was run as 6 doubling dilutions (1:50-1:1600) as single replicates per assay run. The 6^{th} adult serum collected constituted the control, and was run at 2 dilutions, 1:100 (high control) and 1:400 (low control) in duplicate on each plate.

5.6 Lysate Preparation Optimization and Assay Troubleshooting

The experiments described above were carried out with lysate provided by Dr. Paul Scott. Subsequently, lysate prepared in the course of the project performed inconsistently. Great inconsistency between duplicate readings of test sera and especially between standards and controls on different plates was observed (Figure 5.7). Various batches of lysate were utilized to carry out analysis of all samples. Batches of lysate were either harvested on the same day but used to coat plates on different days, or harvested on consecutive days – but were from the same parent cell line. On coating ELISA plates, it was subsequently noted that lysate drying rates varied from one well to the next, as well as from one plate to the next and could have contributed to some of the variation in OD readings noted. The variation in ELISA results could have also been due to the existence of very different concentrations of viral proteins despite the same total antigen concentration.

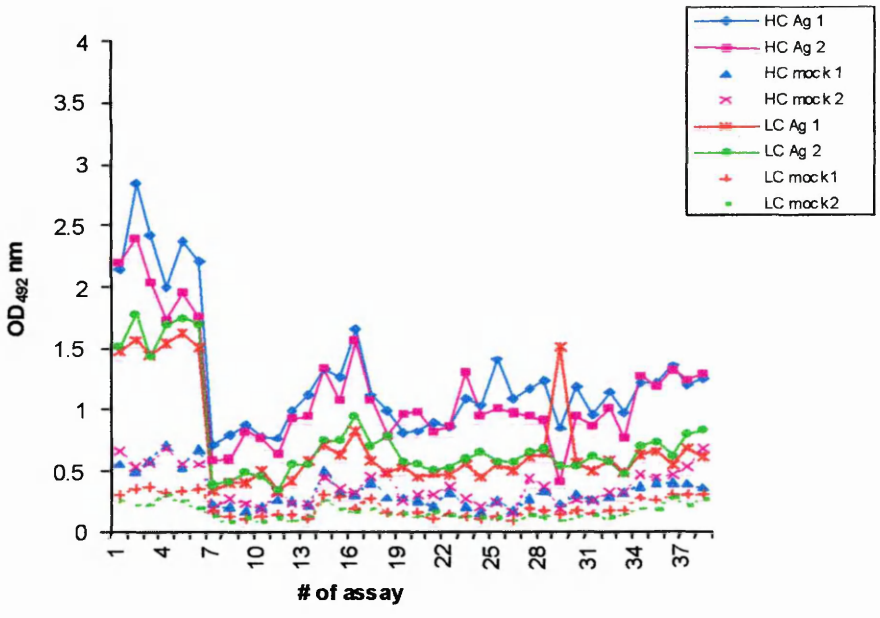


Figure 5.7. Variation between assays for high and low controls together with respective mock/control under old assay conditions (respective Ag and mock/control OD readings indicated by same colour). Hc: high control; Lc: low control; Ag: antigen

Furthermore, the ELISA detects non-native antigens. Despite efforts taken to try and minimize manipulation during lysate production in order to conserve epitope structures, it was plausible that different degrees of conformationally relaxed or otherwise altered epitopes not typical of the virion were produced. Therefore, peptides representing regions of the molecule that are normally buried may have been exposed generating an antiviral response not typical of intact virus or protein and could have added to the variation described.

In an attempt to minimize assay variation, liberation of more viral antigen was carried out. In addition to using the non-ionic detergent NP40 that is known to have a solubilizing effect on cell membrane components (Helenius & Simons, 1975), lysate was disrupted by ultrasonic treatment with a sonicator at an output of 70% amplitude 3X 1min cycles with 1 sec pulse and

1 sec pause, as sonication had previously been shown to increase OD readings (Sarkkinen et al., 1981).

The permissive temperature (a temperature which permits optimal pathogen replication and hence growth) for RSV is said to be around 32°C (Broughan et al., 1997, Schnitzer et al., 1976) and therefore it can be presumed that temperatures greatly above this will be non-permissive or restrictive, leading to diminished virus titres with the resultant production of less Abs. This too could have been a contributing factor to some of the low OD readings observed from time to time. Initially due to logistics, all cell cultures infected with the A2 isolate were carried out at the non-permissive temperature of 37°C, therefore infection was attempted at 32°C and using the sonicated lysate, checkerboard analysis under the initial ELISA conditions was carried out again. This helped increase OD readings. However, different rates of evaporation from plate-to-plate and well-to-well still persisted.

In addition, the presence of Tween-20 in the block was omitted, however it was still retained in the washing buffer as low concentration of detergent are effective components of washing buffers, strongly inhibiting non-specific interactions (http://www.kpl.com/technical/techdocsearch.cfm?tcat=3). PBS was also investigated as coating buffer. Plates were sealed to prevent evaporation, and coating carried out at various temperatures and for varying periods of time *e.g.* 4°C overnight, room temperature overnight, 37°C for 4 hours *etc.* Incubation at 37°C overnight gave higher OD readings.

To further increase the sensitivity of the assay, apart from carrying out routine steps of optimizing the blocking solution, washing and incubation times or testing different plates,

covalent linkages to plate has been suggested overcomes low level attachment of bound molecule which in turn gives low signal (http://www.kpl.com/technical/techdocsearch.cfm?tcat=3). The latter method was not investigated, but new ELISA plates, Nunc-Immuno™ MaxiSorp™ were tested as they comprise surfaces of high and uniform binding capacity (http://www.nalgenunc.co.jp/data/bulletins/06.htm), and in conjunction with the above modified conditions, it appeared that we had achieved inter- and intra-plate consistency. The coefficient of variance within plates varied between 6-7% and between plates was 6%. Moreover, a greater signal: noise ratio and consistently high OD readings for the high control were realized (Figure 5.8). Although there appears to be some decay with time with regards to the high control, this was not noted in the long term quality control of the controls for the full data set. The modified ELISA procedure can be found in section 4.5.2.7.

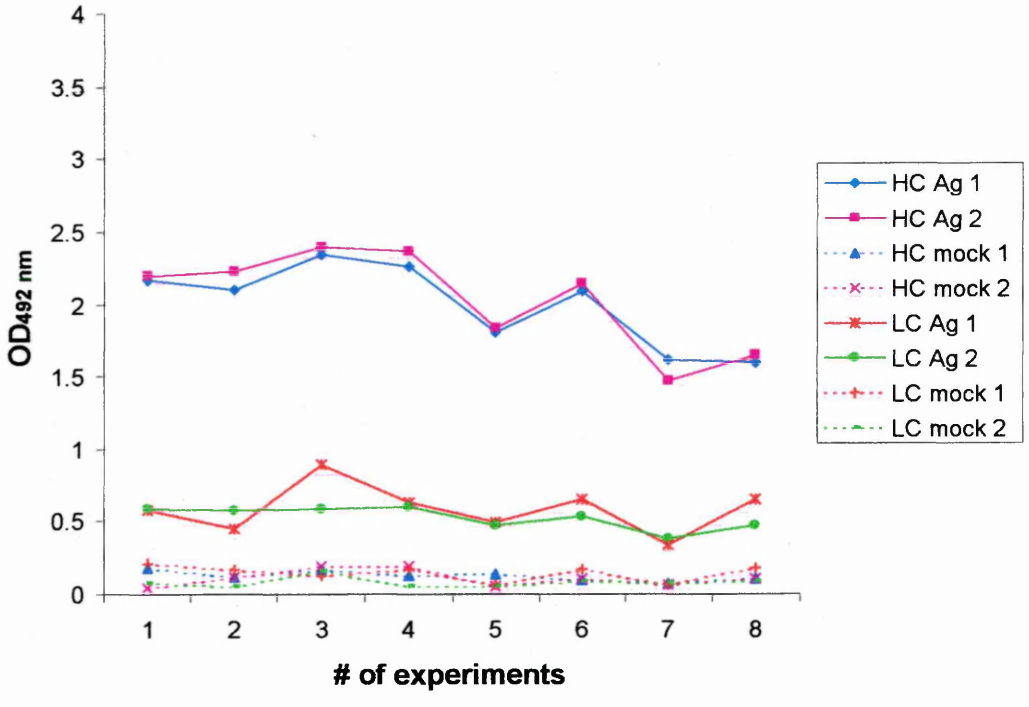


Figure 5.8. Variation between experiments for high and low controls together with respective mock/control under new assay conditions (respective Ag and mock/control OD readings indicated by same colour). Hc: high control; Lc: low control; Ag: antigen

5.7 Discussion

From the checkerboard analysis, the optimal dilution of the capture- solid phase antigen was 1:64, whilst that of the rabbit anti-human IgG conjugate was 1:1000. Test sera were tested in duplicate at a dilution of 1:100. At higher serum concentrations, high background ODs from uninfected tissue culture control wells obscured readings from test wells. Furthermore, the sensitivity of the test was dramatically reduced on testing sera at low titres.

The prozone-like effect observed for some children was perhaps due to the presence of excess analyte or matAb in this case, that caused saturation of both specific and non-specific binding sites during antigen-Ab binding to the inert support (Killeen et al., 1993). When eventually desorbed, it could have attached to signal Ab thus preventing the formation of the 'sandwich' (Selby, 1999) and leading to the hook effect. Steric inhibition could also have occurred as a result of specific immunoglobulin attachment, the presence of non-specific inhibitors in human sera, or both. A 1:100 dilution appeared to capture the log linear phases of a majority of the 3-monthly samples tested and was therefore chosen as the appropriate dilution for assaying samples. Additionally at this dilution, the prozone effect was excluded. Also, as log linear phases of dose response curves for different children were shown to be párallel (Figure 5.6a-b), the use of one dilution was representative of RSV IgG levels. Finally, background ODs remained low and readings were reproducible, although it was sometimes noted that an occasional serum reacted with control cell antigens to a similar extent as with the test RSV cells.

Thus, preliminary results from the assay detected rising titres between paired samples and therefore confirmed recent infection. In addition, both low and high Ab titres in paired

samples were measured by the assay. Dose response curves were observed to be approximately sigmoidal, and decay of matAb occurred over a period of 6 months to a minimal level.

It was decided therefore, that the ELISA had been optimized as far as possible, and hence extensive analysis of cohort samples, which constituted paired sera, 3 monthly and cord sera could be carried out and analyzed at a 1:100 dilution on appropriately coated ELISA plates. Under these optimized conditions, reference serum was to be run in parallel with the test samples, on every plate over a range over of 1:50-1:1600 that captured the log linear portions of dose response curves. Over this range, Abs could be measured at the highest sensitivities. The inclusion of standards allowed standardization of results and took into account both inter- and intra-plate variation possibly caused by the slow deterioration of coating antigen. However, reference serum would be run as single replicates. Controls were assayed in duplicate at 1:100 and 1:400 dilutions respectively.

CHAPTER SIX

Characterization of RSV Strains Infecting Children from a Birth Cohort using Multiplex Polymerase Chain Reaction (mPCR)

6.1 Introduction

Two RSV groups were originally identified on the basis of reactions with monoclonal Abs (Anderson et al., 1985, Gimenez et al., 1986, Mufson et al., 1985). The protoypes of group A were identified as Long (isolated in 1956) and A2 , and for group B as CH-18537 (isolated in 1962) by early investigators (Coates et al., 1963). Later, these groups were shown to be distinct at the nucleotide sequence level (Johnson & Collins, 1989, Johnson et al., 1987).

The G protein is the most variable protein between groups. Amino acid similarity of this protein from a group A and a group B strain was shown to be only 53% (Johnson et al., 1987), with the G protein displaying up to 20% amino acid variability for group A isolates and 9% for group B isolates (Cane et al., 1991, Sullender et al., 1991). It was therefore realized that heterogeneity existed within groups (genotypes or lineages), in addition to the variation earlier noted between groups. Group A genotypes were initially designated SHL1-6 (their corresponding NP groupings are shown in table 6.1) and group B genotypes NP1 and NP3 (Cane & Pringle, 1991, Cane & Pringle, 1992). SHL groupings were designated by sequencing and NP groups by RFLP.

Table 6.1. Summary of SHL groups with corresponding NP groups (Cane & Pringle, 1991, Cane & Pringle, 1992)

SH lineage	NP group	Group
SHL 1, 3, 4	NP 2	A
SHL 2	NP 4	A
SHL 5	NP 5	A
SHL 6	NP 4	A

Stockton et al. (1998) described a multiplex polymerase chain reaction (mPCR) assay and this has proved to be faster and more sensitive than conventional viral culture or antigen detection methods (Coiras et al., 2003). The assay distinguishes between A and B groups and uses a combination of several different primer pairs in the same amplification reaction with the objective of producing different specific amplicons that are easily discriminated between on the basis of their sizes.

We used a modified version of the method by Stockton et al (1998) and N primers as earlier described by Cane and Pringle (1991) to investigate the children in rural Kilifi community for RSV during 4 consecutive epidemic periods (2002-2005). The 4 epidemics occurred as follows: Epidemic 1: 01 Mar-31 July 2002; Epidemic 2: 01 December 2002- 30 April 2003; Epidemic 3: 01 January- 31 July 2004 and Epidemic 4: 01 November, 2004- 30 April 2005. Viruses were characterized into group, and variability within the groups was assessed by RFLP of amplified N gene products.

6.2 Objective

To analyze the strain variation of RSV isolated from NW of children recruited to the birth cohort using the methods of mPCR and RFLP as a means of characterizing the circulating

strain(s) and hence, the molecular epidemiology of RSV in the community during each epidemic. Additionally, the genotypic variation in RSV strains causing infection in the birth cohort (which were generally non-severe) was compared with those causing severe disease requiring admission to KDH.

6.3 Methods

Briefly, the assay has two main stages: firstly, nested mPCR was carried out on extracted RNA to distinguish between RSV A and B, and secondly, a round of N gene PCR was carried out. The final analysis was carried out by RFLP of N gene PCR products and this allowed for delineation of groups on the basis of the NP typing patterns (motifs) or lineages as previously described (Cane & Pringle, 1991, Cane & Pringle, 1992). For a detailed description of the method, refer back to the molecular assays (Section 4.5.3). There were 2 types of NW RSV positive samples as defined below that were identified and analyzed.

6.3.1 Definitions

- Symptomatic IFAT confirmed NW samples were identified during passive and active surveillance and subsequently prompted collection of acute and convalescent sera samples one month apart (Section 4.2.2).
- ELISA confirmed NW samples were identified upon completion of analysis of all sera samples by ELISA. On visual confirmation of rising titres within epidemics in which no positively confirmed IFAT results existed, all NW samples collected from the beginning of the epidemic up to the peak of the rise in titre were typed.

Figures 6.1 shows a schematic of the points where NW samples had been collected during ARI surveillance, and found to be IFAT negative. These were later retrieved from archives for further testing by mPCR.

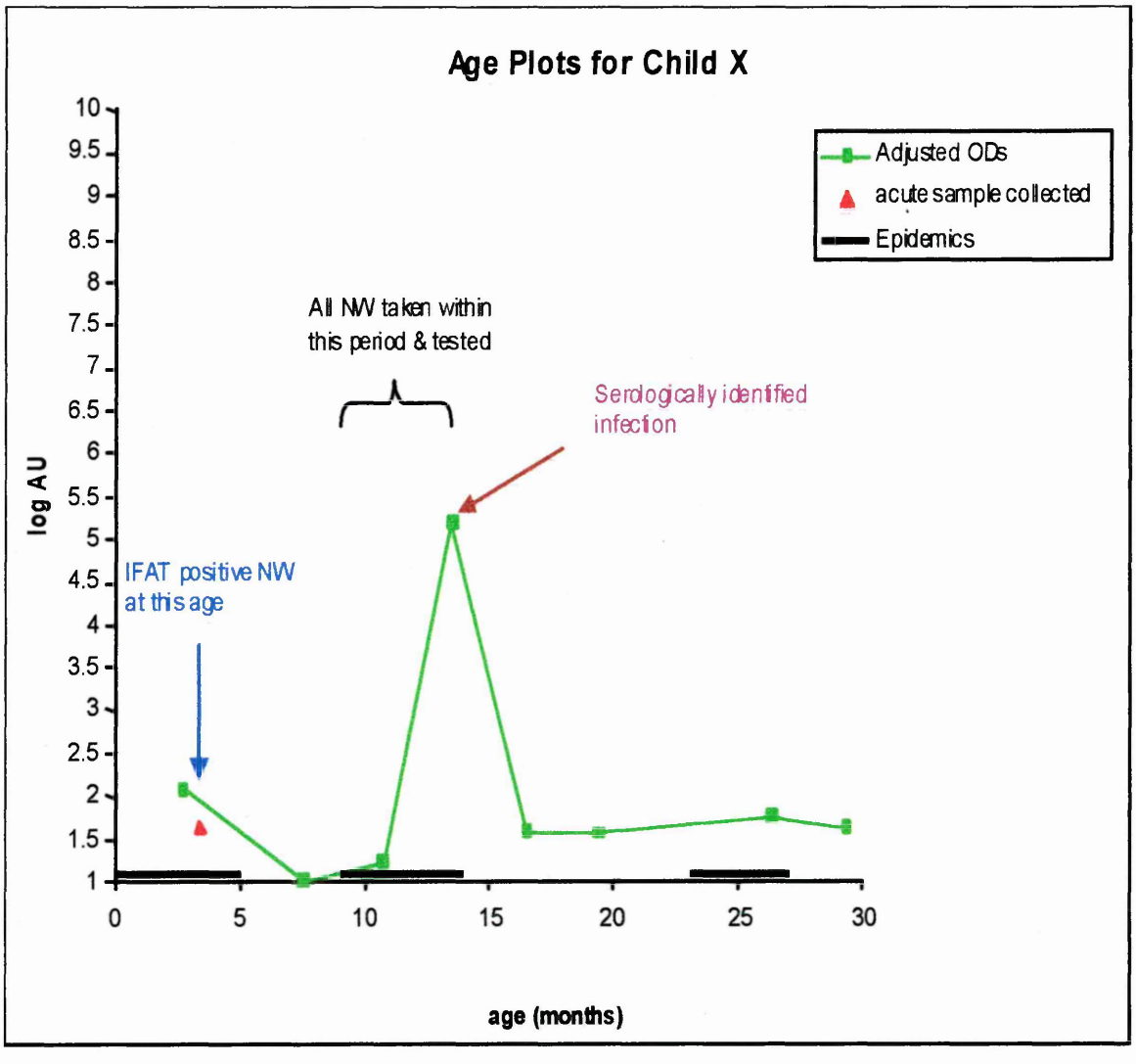


Figure 6.1. A schematic indicating points at which NW samples (those that were IFAT positive and those collected weekly in epidemic period between 2 consecutive 3 month samples that showed ELISA confirmed serological response) were identified for further analysis of RS viruses by mPCR

6.4 Results

6.4.1 Clinical specimens

During the entire 4 years of follow up, which amounted to a total of 926 child years of observation, of the 8717 episodes of ARI requiring a NW, 97% (8493, Appendix IX) were taken. The remaining 3% were not taken due to various reasons including refusals by the infant's mother, discomfort of the child and failure to collect adequate sample after 2 attempts. NW samples were processed as soon as possible upon being brought into the laboratory. This allowed for feedback to the field workers for follow-up. RSV project laboratory staff tested these samples. A total of 457 (5%) and 7,905 (93%) samples were identified as being positive and negative by IFAT, respectively, and the remainder equivocal (110) or untested (21) (Appendix IX). Subsequent screening of birth cohort sera by anti-RSV ELISA confirmed a majority of these antigen positive cases (IFAT confirmed serological responses, IFAT-CSR), and picked up additional IFAT negative but serologically positive samples (ELISA confirmed serological responses, ELISA-CSR). This will be discussed further in chapters 7 and 8. To further investigate the the exact date of infection and virus strain responsible for seroconversions that were noted between 2 consecutively collected 3 month samples, 80 NW samples collected around this time (Figure 6.1) were also analyzed by PCR.

Most cases of RSV infection observed from 2002 to 2005 were diagnosed in the first half of each year, with the peaks of the epidemics occurring at varying times, *i.e.* May/June 2002; February 2003, April/May 2004 and February 2005 respectively.

6.4.2 Processing of IFAT positive NW specimens

68% of IFAT positives yielded mPCR products. IFAT positive/mPCR positive samples were further analyzed by molecular techniques for genotype. A typical gel depicting bands detected from IFAT positive NW samples is shown in Figure 6.2. Both RSV A and B were identified.

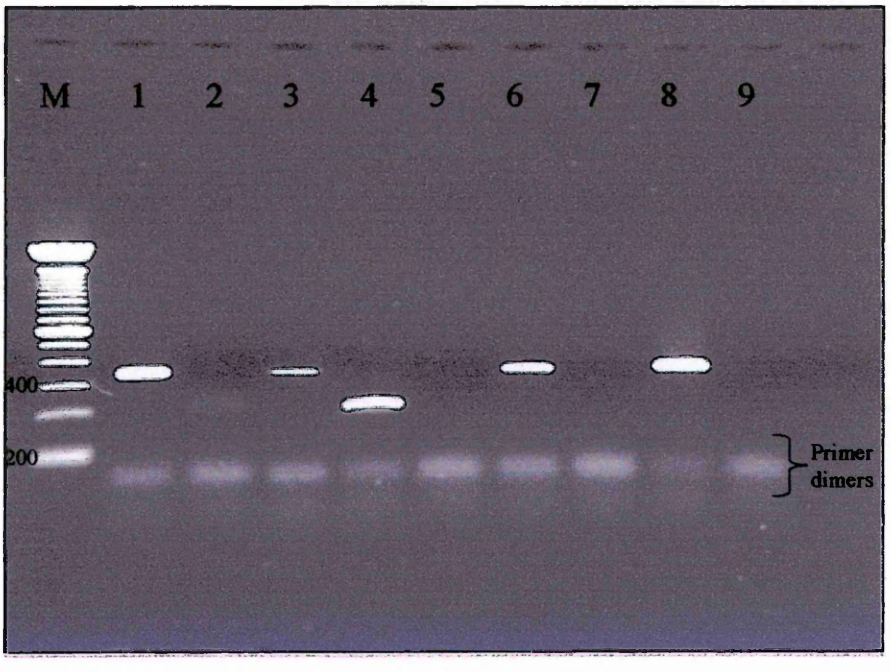


Figure 6.2. An example of a typical gel showing RSV typing of IFAT positive NW clinical specimens by mPCR. Lanes 1-7, clinical specimens; lanes 8 and 9, are positive and negative controls respectively. Lanes 1, 3, 6 and 8, RSV group A; Lanes 2 and 4 RSV group B. Lane M, molecular size marker. Numbers on the left are in base pairs.

Following the screening process, N gene PCR was run on 1st round amplicons/products of positively identified NW samples. RFLP was carried out on the PCR products for further identification of infecting genotype. The resulting banding patterns obtained following digestion by the five restriction enzymes, Hind III, Pst I, Bgl II, Hae III and Rsa I comprised the NP typing patterns (Table 6.2).

Table 6.2. N gene fragment restriction digest patterns (Cane & Pringle, 1991)

NP group	Enzymes (cut = +; Uncut = -)					RSV A or B type
	Hind III	Pst I	Bgl II	Hae III	Rsa I	
NP1	-	-	-	-	+	B
NP2	-	-	-	+	+	A
NP3	-	-	+	-	+	B
NP4	-	-	+	+	+	A
NP 10	-	-	+	+	+*	A
NOVEL	-	-	-	+	-	A

The table shows how the 5 restriction enzymes loaded in this order on the gel i.e, Hind III, Pst I, Bgl II, Hae III and Rsa I cut the N gene PCR products for the NP types detected in this study.* depicts digestion by Rsa gives 3 bands compared to the 2 bands formed following Rsa digestion in NP4 pattern.

A novel banding pattern was observed in epidemics 1 and 4. This banding pattern is shown in Figure 6.3 below. This figure shows typical RFLP results on a selection of positive samples identified in Figure 6.2; the banding pattern for the positive control is also shown. Typical NP4 pattern is shown for 2 patients in Row A, lanes 1-5 and 7-11. The positive control was also an NP4 banding pattern, Row B lanes 13-17. Typical NP3 pattern for 2 patients is shown in Row A lanes, 13-17 and Row B, lanes 7-11. The novel banding pattern for 1 patient is seen in Row B, lanes 1-5.

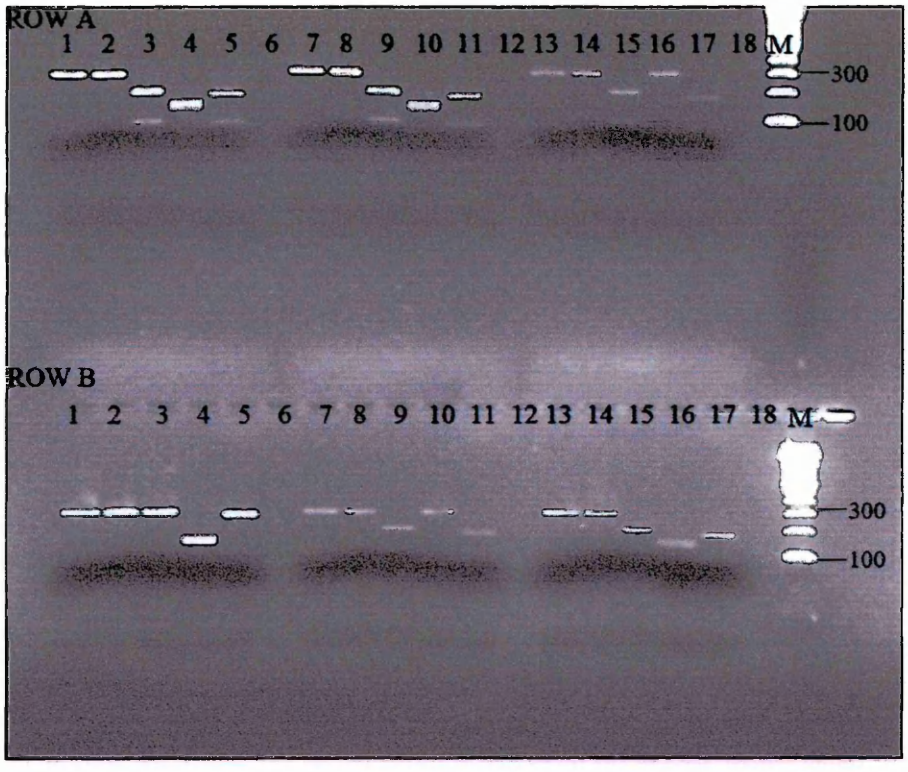


Figure 6.3. Gel showing NP typing patterns obtained following N gene PCR and RFLP of positive nested PCR samples (positive 1st round amplicons from samples and control identified in Figure 6.2). Row A, lanes 1-5, 7-11 and 13-17 are restriction of PCR products from individual patients of banding patterns NP4, NP4 and NP3 respectively. Row B, lanes 1-5, 7-11 are individual patients of banding patterns Novel and NP3 respectively, and lanes 13-17 depicts the control, NP4 banding pattern. The enzymes used in lanes 1, 7, 13 was Hind III; lanes 2, 8, 14 was Pst I; lanes 3, 9, 15 was Bgl II; lanes 4, 10, 16 was Hae III and lanes 5, 11, 17 was Rsa I. Lane M, molecular size marker. Numbers on the right are in base pairs.

6.4.3 Distribution of variants during epidemics

The following sections describe RSV variants from IFAT positively identified NW samples that were characterized in each epidemic.

6.4.3.1 Relative prevalence of RSV groups

Ninety-eight percent (271/276) of isolates identified from infants, during epidemic 1 were from RSV group A. In the next epidemic, RSV A and B co-circulated in relatively equal proportions, whilst in epidemics 3 and 4, 100% (252/252) RSV A and 98% (122/124) RSV A were detected respectively as shown in Table 6.3.

6.4.3.2 Strain typing by RFLP

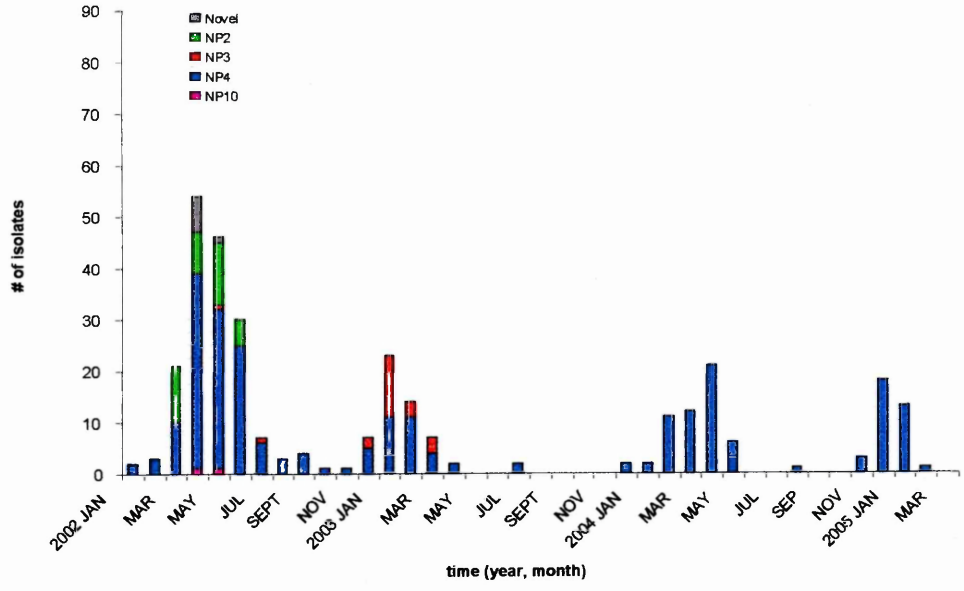
The results of N gene RFLP typing further demonstrated that the epidemics were not homogeneous (Table 6.3). The majority of group A samples were genotyped as NP4, and NP3 comprised the majority of group B. Group A samples consisted of 4 N gene banding types all of which were present in the first epidemic, with both group B types being observed in epidemic 2. During the 1st, 3rd and 4th epidemics, NP4 was the most dominant strain, whilst NP4 and NP3 co-circulated in equal proportion in the 2nd epidemic. The relative proportions of both group A and B strains are shown in Table 6.3. The proportions of RSV strains isolated from cohort infants were compared with those isolated from inpatients at the KDH (Figure 6.4).

Table 6.3. Numbers (proportions) of RSV A and B, and NP types in each epidemic isolated from cohort infants

Epidemic #	Total NW tested	RSV-A NP Pattern					RSV-B NP Pattern			Total Positive
		NP2	NP4	Novel	NP 10	Total RSV-A (%)†	NP1	NP3	Total RSV-B (%)†	
1	540	64	195	10	2	271 (98)		5	5 (2)	276
2	228		84			84 (52)	2	76	78 (48)	162
3	444	1	251			252 (100)				252
4	337		102	20		122 (98)		2	2 (2)	124

†Total percentage values for RSV A and B N gene based on proportion of the total N gene RT-PCR positive samples (final column) rather than total samples tested

a) Inpatient: 2002- 2005



b) Community (cohort): 2002-2005

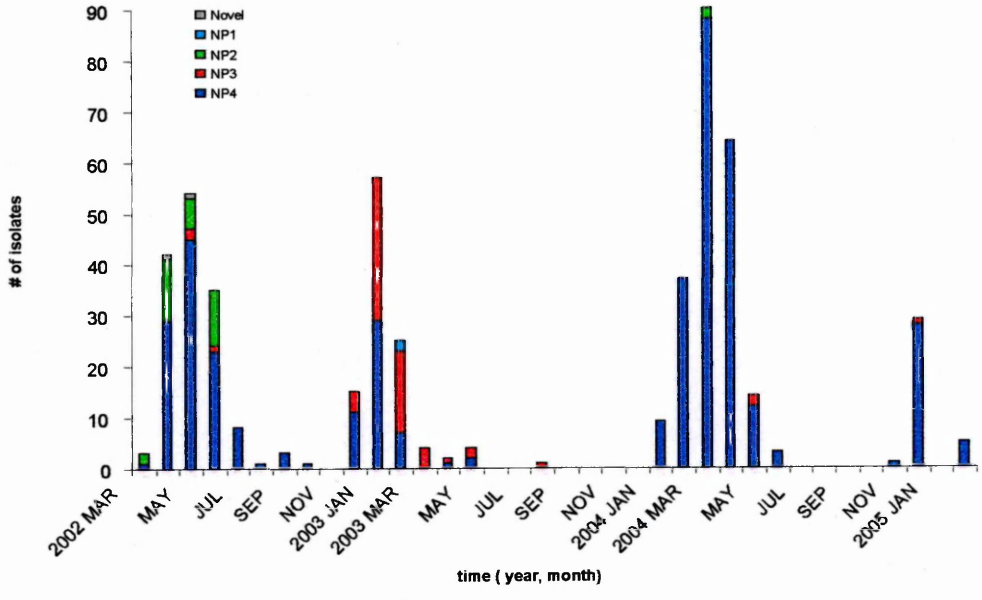


Figure 6.4. Proportions of NP typing patterns in Kilifi District in KDH inpatients (a) and the community, birth cohort (b)

6.4.4 The effect of strain variation on disease outcome

It was noted that disease severity was not affected by circulating strain. With respect to infants recruited within the birth cohort, the same strain was seen to cause both URTIs and LRTIs as noted in Table 6.4, however, not much can be deduced on the effect of strain variation on disease outcome due to the small number of children who went on to experience RSV-associated pneumonia.

Table 6.4. RSV-associated disease with strain as depicted by the N digest pattern in children < 6 mo of age

N digest pattern	Pneumonia status			
	No/URTI	Mild	Severe	Very severe
New	0	1	0	0
NP2	10	2	4	0
NP3	5	2	1	0
NP4	16	17	23	1
Total	31	22	28	1

It was noted NP4 was the most dominant strain in infants, aged 0-4 months, recruited in the 1st round (cohort 1). NP2 and NP3 were also identified, but at reduced levels. In the following seasons NP3 and NP4 co-circulated in both sets of children. However, NP3 was observed to infect all age groups (cohort 1: 0-2 months and cohort 2: 6-13 months) whilst NP4 was restricted to older children of cohort 1. As more epidemics were experienced, NP4 was seen to be the most abundant isolate in all age groups, children from both phases of the cohort being affected (Figure 6.5).

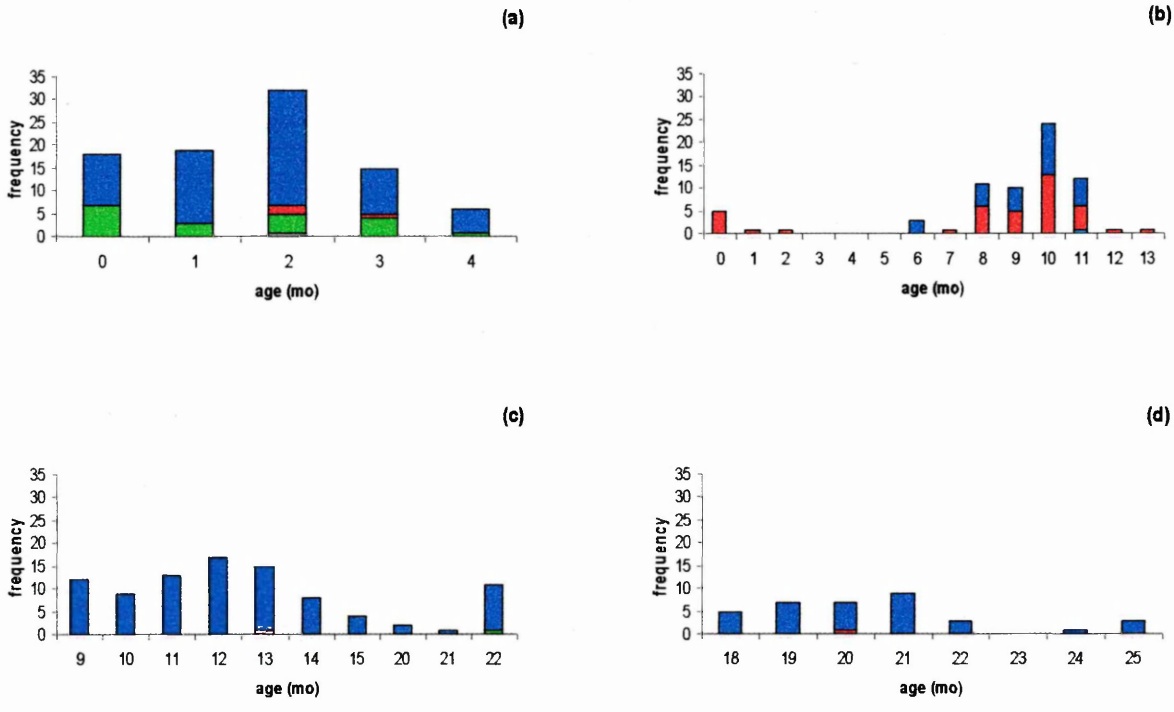


Figure 6.5. Strain variation with age for children recruited to both phases of the cohort throughout the 4 epidemic periods. (a) depicts epidemic 1 (b) epidemic 2 (c) epidemic 3 and (d) epidemic 4 (d), respectively. The different strains identified were New ■, NP4 ■, NP3 ■, NP2 ■ and NP1 ■

6.4.5 Processing of NW specimens identified from an ELISA-CSR

Only NW samples collected, within an epidemic, following identification of ELISA-CSR between 2 consecutive 3 month samples (see Figure 6.1) were tested. Eighty NW samples were identified from these defined periods and further tested by mPCR. Despite repeated attempts, none were shown to be positive.

To further investigate the detection limit of IFAT positive samples by RT-PCR as a proxy for detection of amplicons in ELISA-CSR positive samples, 4 known IFAT positive NW samples were tested in parallel with 1 RSV A HEp-2 culture positive sample. Five 10-fold serial dilutions were carried out on the 4 samples, with five 5-fold serial dilutions being carried out for the positive control. The results are illustrated in Figure 6.6. Some faint bands could be

seen in the diluted culture sample up to a dilution of 1:3125; the clinical specimens were positive at dilutions of 1:10 (3 samples) or only neat.

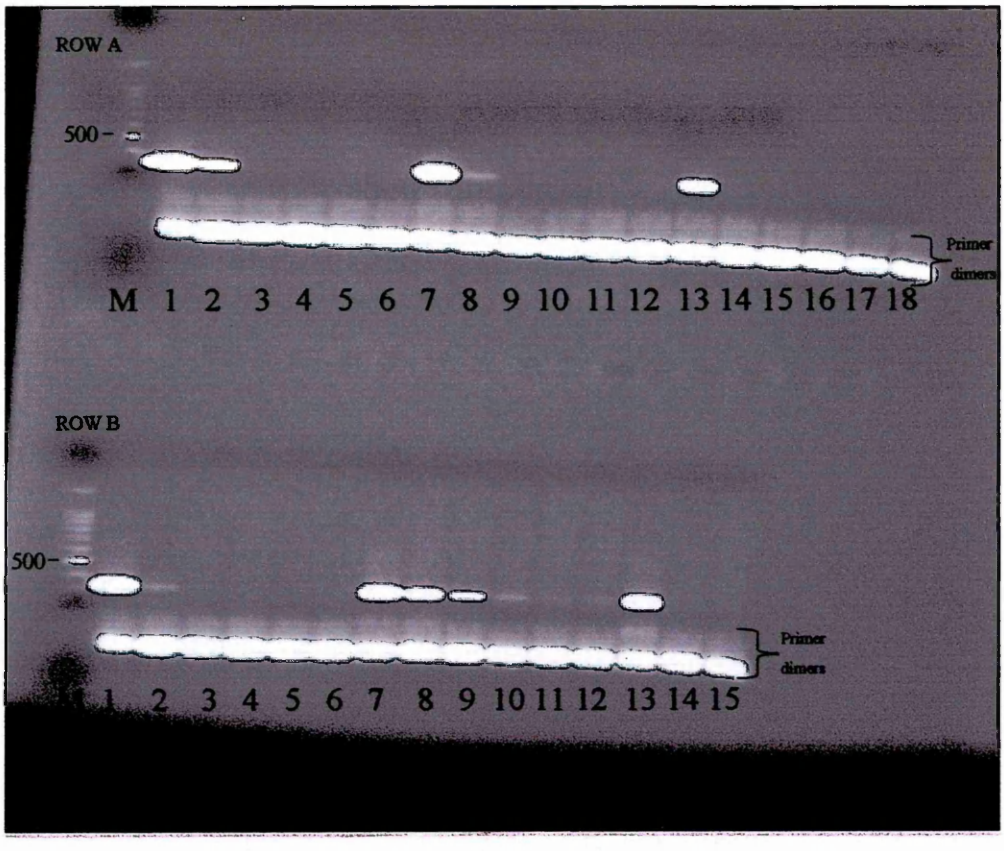


Figure 6.6. Serial dilution of IFAT and culture positive samples as a means of working out detection limit. Row A, lanes 1-6, lanes 7-12, lanes 13-18 and Row B, lanes 1-6 are 4 individually serially diluted positive clinical specimens. Row A, lanes 1, 7, 13 and Row B, lane 1 depicts the undiluted sample, successive lanes are 1:10, 1:100, 1:1,000, 1:10,0000, and 1:100,000 dilutions respectively of that sample. Row B, lanes 7-12 is RSV A HEp-2 culture positive; in which Row B, lane 7 is the undiluted culture, successive lanes depict 1:5, 1:25, 1:125, 1:625 and 1:3125 dilutions respectively. Row B, lane 13 is positive control; Row B, lanes 14-15 are the negative controls. Lane M is molecular size marker. Numbers on the left are in base pairs.

6.5 DISCUSSION

The study presented here was designed to assess RSV variability over a period of 3 years of follow up per child in a rural community in coastal Kenya. Analysis of the diversity of RSV was monitored by a birth cohort and compared to in-patient samples. The samples analyzed were collected from infants or children exhibiting ≥ 1 signs of acute cough, difficulty in breathing or nasal congestion/discharge, observed or elicited by the history from the preceding week. As there was a lack of infrastructure initially, it was not practical to culture viruses. It should also be noted that although RFLP analysis proved to be relatively easy to perform, it has been said that the genotypes obtained by this method provides limited information (Kuroiwa et al., 2004). G gene analysis by the method of DNA sequencing in contrast, would give a more definitive identification of RSV genetic variability within groups. This method however had not yet been set up in Kilifi, and as such, G genotyping and sequencing were not undertaken. Despite this, the mPCR still proved useful in facilitating molecular analysis of available clinical specimens in-house allowing for characterization of RSV circulation patterns in communities as previously described (Mufson et al., 1988, Sato et al., 2005).

The nested RT-PCR was found to be reasonably sensitive (detecting up to 68% of IFAT positive NW samples). The 32% of samples not detected however, implies that the circulating genotypes were most likely underestimated. Additionally, the tendency of RSV B being less likely to be detected than RSV A, could have accounted for part of the 38% negative samples and thus may have been systematically biased. Furthermore, the assay was noted to be specific, *i.e.* there was no cross-reaction with non-RSV specific primers *i.e.* flu primers. The presence of five primer sets within the reaction mixture, did not compromise the RT-PCR assay detecting all virus types tested. This allowed for the classification of RSV to groups,

when archived specimens, frozen within 24 hr from collection, were tested. Viruses were further characterized by RFLP of amplified N gene cDNA products revealing additional genetic variability within each group, with 3 to 5 restriction patterns observed during each period.

By virtue of the existence of RSV strains, outbreaks caused by genetically distinct strains can follow an outbreak caused by a single strain (Hall, 1999, Imaz et al., 2000, Walsh et al., 1997) as was noted in epidemic 3 in which the outbreak genotype was NP4, following an epidemic of multiple genotypes (NP4 and NP3). Furthermore, it was observed that there was hardly any NP3 (group B) in the first season, whilst in the subsequent season, there were more infections being caused by this strain. Similar variability was previously described in other studies (Anderson et al., 1991, Cane et al., 1994, Hendry et al., 1989). Our results therefore provided further support for genotype designation by RT-PCR methods as an effective tool for the characterization of circulating RSV within communities, as previously observed (Anderson et al., 1991, Cane, 2001, Cane et al., 1999, Hall et al., 1990, Imaz et al., 2000, Loscertales et al., 2002, Roche et al., 2003, Venter et al., 2002, Wang et al., 1995, Wilcox et al., 1991).

From our study over the 4 year time span, no significant differences were observed in strain distribution between community cases and severe inpatient cases, the same strain seen to cause both URTIs and LRTIs. Previously, Coiras et al. (2003) performed analyses on RSV variability on isolates from hospitalized babies. They argued that it had not been proven that these samples were entirely representative of the virus that is generally circulating in the community. However, it appears from our study, data with regards to isolates from

hospitalized infants, whom constitute a very selective group of patients, can be extrapolated back to the community and epidemics as a whole.

Analysis of NW samples from all 4 epidemics revealed that RSV A (NP4) was the predominant group as previously described (Morgan et al., 1987, Peret et al., 1998, Venter et al., 2001, Rajala et al., 2003). Genotype variability was described for both group A and B RSV, although to a lesser extent for group B. Additionally, both RSV groups were observed to co-circulate in the 2nd epidemic. The genotypes identified in this epidemic were NP3 and NP4. Co-circulation of RSV isolates have been previously documented by numerous authors (Cane et al., 1999, Coggins et al., 1998, Mufson et al., 1988, Peret et al., 1998). This predominance of NP4 in all epidemics may have been an artefact due the low sensitivity of the nested RT-PCR noted above, or its inability to consistently detected RSV B. However, when RSV B was detected, the actual prevalence of strains varied, and due to these observed differences, it is suggested that separate and independent circulation of group A and B RSV isolates occurs (Hendry et al., 1986). Moreover, this genotype displacement or shift in RSV strains is consistent and thus suggests that it is an important feature of the epidemiology of RSV infections. A higher sensitivity of detection of RSV in clinical samples could be obtained by a combination with additional techniques, such as virus cultivation.

During the first epidemic that comprised infants recruited to the 1st cohort, it was noted that NP2 and NP4 strains were common, with NP3 strains circulating at very low levels (Figure 6.5a). In this regard, matAb levels in the community to these abundant strains were probably boosted (herd immunity), leading to what seemed like effective protection for children born and recruited to the 2nd cohort as none of the infants < 6months of age in the subsequent

epidemic season experienced infection caused by either NP2 or 4 (Figure 6.5b). This was as similarly noted in previous studies (Ward et al., 1983) where the authors illustrated that mothers of protected babies had significantly higher levels of a specific virus-specific protein that was also described in infant sera. It was therefore possible that the circulating strain in the previous epidemic comprised this virus-specific protein, culminating in boosting of specific maternal titres, and thus an efficient maternal-foetal transport mechanism allowed for the protection of these babies from strains displaying a protein that offered cross-protection. The role of RSV-specific matAbs will be further explored in chapters 7 and 8.

These temporal patterns in RSV group dominance and genotype predominance displacement have been observed to vary between locations (Cane et al., 1991, Cane & Pringle, 1992, Hall et al., 1990, Loscertales et al., 2002, Peret et al., 1998, Venter et al., 2001). At the local level, it might be that the varying patterns of dominance are suggestive of immunity, associated with the presence or absence of matAbs which in turn dictates susceptibility of the child with respect to infection by homologous or heterologous genotypes. It is likely that the multiple lineages seen during epidemics emerged under the influence of immune responses in the different communities (Cane & Pringle, 1995) leading to an inadequate immune response due to virus evasion of the immune system. The impact of antigenic diversity on RSV epidemiology is not fully understood, but may in part explain susceptibility to re-infection throughout life and yearly variation in the severity of epidemics within communities (Cane et al., 1994). In this study, the same genotypes were identified both in the community and in-patients. Given the confusing relationship between Ab status, strain variation and re-infection, it is clear that additional longitudinal studies are required for investigation of the transmission dynamics of RSV as early information about RSV variants causing disease might help the

clinician decide on appropriate therapy, especially in younger infants. This is further discussed in the chapter 7, in an attempt to try and assess the dynamics of maAbs in the first year of life, as well as to characterizing the association of the presence or absence of matAb and acquired immunity with circulating strain in Kilfi.

None of the 80 NW samples collected following ELISA-CSR gave any RT-PCR products despite repeated attempts. Plausible explanations for the negative PCR results could be due to the presence of low viral copy number in clinical sample, presence of viral inhibitors and poor specimen handling or collection (Henrickson, 2004). The most likely explanation however is that the presence of very low levels of virus in secretions, in addition to the low detection limit observed for positive samples (Figure 6.6). Furthermore, the series of 'freeze- thaw' steps that occurred between nasal sample collection and RT-PCR processing, could have contributed to the degradation of low-level RNA. The detection limits of the RT-PCR could be improved further, especially in patient groups exhibiting low titres of RSV, and in environments in which optimal conditions of sample collection, storage and transportation are not available (O'Shea & Cane, 2004) by the use of real-time PCR. Therefore, real time PCR may therefore be suitable in aiding detection of low levels of virus as in this case.

In summary, the data obtained from this study provides additional evidence of variation in RSV strains that is probably an important feature of the epidemiology of RSV infection (Peret et al., 1998). There appears to be yearly replacement of the most common genotype, the genotypes predominating in one year usually becoming less abundant the following season. The mechanism that accounts for this however, still remains unclear. It has been suggested that the combination of local factors e.g. herd immunity to the previous circulating/prevalent

strain, and possible fitness of the circulating virus, together with global spread have an impact on circulation of strains in a community (Madhi et al., 2003, Peret et al., 1998). Certainly the relationship between viral genetic variation, infection/disease and transmission will add to this complex relationship (White et al., 2005). In this respect, successful variants may persist and remain dominant for more than one season as seen in the years 2004 and 2005 in Kilifi where NP4 (RSV A group) predominated. Also, as group A viruses were seen in all 4 epidemics, the higher variability displayed in this group may have an added advantage allowing for its higher worldwide predominance (Anderson et al., 1991, Cane et al., 1994, Hall et al., 1990, Imaz et al., 2000). Finally, this study highlights the fact that RSV epidemiology is similar to those taking place around the world, although some distinct clusters were described (Scott et al., 2004).

CHAPTER SEVEN
The level and duration of RSV-specific maternal IgG and its association with risk factors in infants in Kilifi, Kenya

7.1 Introduction

Current knowledge and possible limitations, with respect to RSV-specific matAb levels and the characteristics of the loss of matAb, are described in chapter 3. Briefly, infants acquire transplacental matAb from the 28th week of gestation (chapter 3). At delivery, cord blood levels of IgG (both total and specific IgG) reflect those of the mother. Maternally derived specific Abs have been demonstrated to provide protection from infectious diseases including RSV (Brandenburg et al., 1997, Caceres et al., 2000, Glezen et al., 1981, Ogilvie et al., 1981, Roca et al., 2002). Various factors have also been identified that affect matAb levels and hence the efficiency of placental transfer. These factors were previously discussed (Section 3.2.3) and include: newborn GA, maternal malaria, maternal nutritional status, maternal exposure to RSV (this indirectly can be extrapolated to when the child is born), maternal HIV status (if the child's status at birth is known, one can then extrapolate back to the mother's status). In the present study, the effect of the following risk factors on matAb decay and cord titres were investigated using multiple linear regression analysis (Ab titres on age variable in days): cord blood levels (divided into quartiles), birth order (divided into 3 levels, 1, 2 and 3 being representative of 1st- 3rd birth, 4th-6th birth and > 6 births respectively), birth weight level (divided into 2 levels, less than and greater than 2.5kg) and birth in or out of an epidemic period.

The durability of the immune response was investigated through changes with age in proportion with total IgG and this allowed for the decay rate or $T_{1/2}$ of RSV specific matIgG (this being the time taken for Ab concentration to decrease to half) to be calculated.

7.2 Objectives

The objectives of this chapter are:

- to describe the main characteristics of the study group;
- to investigate cord blood levels of children who experienced an infection and those who did not under 6 months of life;
- to define a cut-off value for RSV seropositivity for the whole population;
- to present a simple model for the rate of decay of RSV-specific matAbs;
- to fit a simple model to age prevalence data; and
- to investigate the effect of risk factors on both cord titres and rate of decay.

7.3 Definitions

- Ab titres were reported as standardized arbitrary units (AU). As the concentration of standards was unknown, the lowest dilution, 1:50, was arbitrarily assigned a value of 1000 AU and the remaining diluted standards appropriately designated.
- Seroconversion was defined in two ways (i) as at least a 2-fold (0.3 log AU), and (ii) as at least a 4-fold (0.6 log AU) or greater rise in titres between 2 samples collected consecutively.
- IFAT confirmed serological responses (IFAT-CSR) were defined as seroconversions occurring between acute and convalescent sera collected approximately 1 month apart on identification of IFAT positive NWs.

- ELISA confirmed serological responses (ELISA-CSR) were defined as seroconversions occurring between two consecutively collected samples that included both 3 month and acute/convalescent sera.
- Seropositivity was defined as an Ab titre of $\geq$1.495 log AU.

The matAb prevalence and the effect of risk factors were thus assessed on the seropositive population up to 6 months of life. To ensure that only matAbs were measured, infants with any evidence of IFAT-CSR or ELISA-CSR at either seroconversion levels were excluded and the 'pristine' rate of decay for the seropositive population, together with effect of risk factors calculated.

7.4 The analysis procedure

This involved the following steps:

(1) Undertake regression diagnostics as a means of ensuring that the model chosen fitted the population.

(2) Identify further outliers by plotting histograms of gradients of children under 6 month of life (population to be used in calculating RSV-specific matAb decline).

(3) Establish cut-off values of seropositivity in the population, and confirm validity of these values by investigating their placement in frequency distribution plots.

(4) Investigate duration of seropositivity at a cut-off level of 1.5 (defined from the assay and 28% of the population was seronegative) and 2.0 (where 50% of population was seronegative).

(5) Calculate 'pristine' rate of decay of RSV matAb by omitting children who experience any infection- both IFAT- and ELISA-CSR in the first 6 months of life. This gave 2

groups- infected and non-infected populations that were subsequently investigated together with the association of possible risk factors using,

a. Student's t-test

b. Linear regression analysis to control for mutiple risk factors and confounders

(6) Investigate cord blood titres in the 2 above groups using

a. Student's t-test

b. Linear regression analysis

7.5 Methods/Statistical Analysis

All data analysis was undertaken using Stata 9.0™. The concentration of RSV-specific Abs (in AU) in infants was determined using a modified ELISA method (Wilson et al., 2000) as earlier described (section 4.5.2.5). The data are highly positively skewed and as a result all analyses were carried out after the transformation of anti-RSV Ab serological levels from arbitrary units (AU) to $\log_{10}$ AU. All log values are to the base 10 unless otherwise indicated.

A cut-off value for seropositivity was determined by the following two methods below:

(1) From observations of Ab decay curves of individual infants: it was observed that matAb levels reached a minimal level by 5-6 months of age. Therefore, the mean of Ab titres ± 2SD within this age bracket for individuals in the population who did not experience any IFAT positively identified infection in the first 6 months was calculated.

(2) From the ELISA assay: this was calculated as the point at which the assay could detect the highest titre without specificity being compromised. Pooled adult sera (standard)

were assayed 4 times in duplicate over a dilution range of 1:25 to 1:51200 in 2-fold steps.

Passively acquired IgG is subject to an exponential decay rate (Sato et al., 1979). Therefore, RSV-specific matAb decay was calculated using the linear regression model. Several assumptions underlie the linear regression model and have to be taken into consideration before analysis. They include the following:

(1) Each observation should be independent of the others.

(2) The values of the outcome variable, Ab titres in log AU follow a Normal distribution.

(3) Variability in Ab titres (log AU), as assessed by the variance or standard deviation, should be the same for each value of age.

(4) The relation between the 2 variables, Ab titres (log AU) and age should be linear.

(5) There is homogeneity of variance or the error of the variance is constant (homoscedasticity).

Non-violation of these assumptions of regression was investigated using regression diagnostics. With regards to modeling the parameters (Section 3.3 for more details) with Ab titres (log AU) as the response variable and age under 6 months as predictor, linear regression was first undertaken on the whole data set, then this population was split into 2: non-infected (those who did not experience any infection, IFAT- or ELISA-CSR, infections under 6 months of age) and infected (those who experienced at least 1 infection, IFAT- or ELISA-CSR, infection under 6 months of age). Infections were defined using both seroconversion levels, 2- and 4-fold change in Ab titre. Furthermore, analysis was carried out on the entire population, which included both the seropositive and seronegative populations (so as not to bias the

outcome). However, with regards to the seronegative population, Ab titres below 1.459 log AU were adjusted to 1.224 log AU (the mean of the seronegative population, Appendix XIa).

Initially data were analyzed independent of possible hierarchy within the data *i.e.* 1-level models where measurement error was the only possible source of uncertainty. However, as there were multiple measurements on individuals, there was a possible lack of independence between repeated observations. We attempted to account for this clustering by using random effects data regression models (XT models, STATA™ 9.1 www.stata.com). Finally, as there is a significant dependence of Ab titres on age, as well as on other risk factors, a multiple linear regression analysis was carried out to examine whether their effects significantly improved the model fit. The differences were further investigated in the under 6 month population using the Student's t-test, the level of statistical significance being set to $P=0.05$. The main variables (all categorical) studied inclusive of age were: (i) cord levels, (ii) weight at birth, (iii) whether the child was born in or out of an epidemic and (iv) birth order of child. All main effects of these variables were examined in a forward regression model. During the modelling process, variables that did not substantially ($P>0.05$) increase the fit of the model based on an increase in adjusted R^2 (goodness of fit) were omitted.

All results reported are for the seropositive (as defined above, Ab titres $\geq$ 1.495 log AU) population. Two-fold seroconversion levels will be compared to the commonly reported 4-fold level.

7.6 Results

7.6.1 Descriptive Analysis of study population

From the 503 infants who were analyzed, a total of 361 cord bloods, 2,777 three monthly sera samples, 295 acute and 272 convalescent blood samples were collected.

Table 7.1. General characteristics of the cohort

Characteristics	Frequency	Percent
Sex: Male	314	49.45
Female	321	50.55
Weight at birth (kg): < 2	27	4.77
2-3	270	47.70
3-4	261	46.11
> 4	8	1.41
Birth order: 1st	142	28.7
2nd	105	21.3
3rd	74	15.0
4th	73	14.8
5th	41	8.3
> 5	59	11.9

7.6.2 Determination of cut-off for seropositivity

From observations of Ab decay curves of individual infants it was observed that Ab titres reached a minimum level at 5 months. Therefore, the average Ab titres at 5 months for the population of infants who did not experience any infection within the first 6 months, was calculated as 1.55 (±0.47) log AU. The high and low cut-off values were calculated as being 2.49 and 0.61 log AU respectively. However, at this cut-off level for seropositivity, the negative and positive sera could not be clearly distinguished.

From the ELISA assay, the linear portion of the standard curve was identified as occurring between dilutions 1:50 (arbitrarily assigned 1000 units, or AU) and 1:1600 (31.25 AU) as

shown in Figure 7.1. We therefore made the assumption that 31.25 AU (1.495 log AU) is about at the lower limit of sensitivity of the assay and therefore took this as the cut-off for seropositivity. This is the cut-off value that is used throughout the analysis, unless otherwise stated.

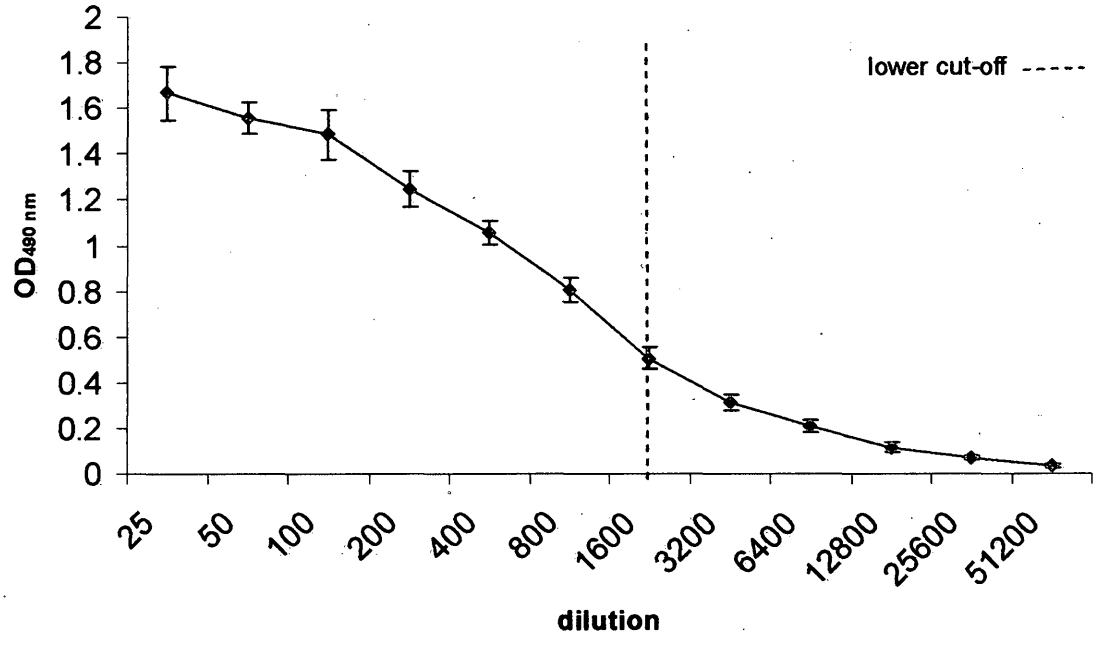


Figure 7.1. Two-fold serial dilution carried out on pooled adult sera lower threshold cut-off indicated. Each dilution was carried out in duplicate and the experiment carried out in triplicate. The error bars denote standard deviation.

7.6.3 Frequency distribution of RSV-specific antibody titres

The cut-off for seropositivity from the ELISA assay was confirmed from the bimodal frequency distribution of Ab titres of all sera (Figure 7.2a). Additionally this seropositive cut-off was investigated by age group (Figure 7.2 b-c). The frequency distribution of Abs is approximately a log-normal distribution. It was noted that the major region of RSV matAb decay occurred below 6 months of age. The higher and increasing proportions of infants with acquired RSV-specific Abs occurred after 12 months of age.

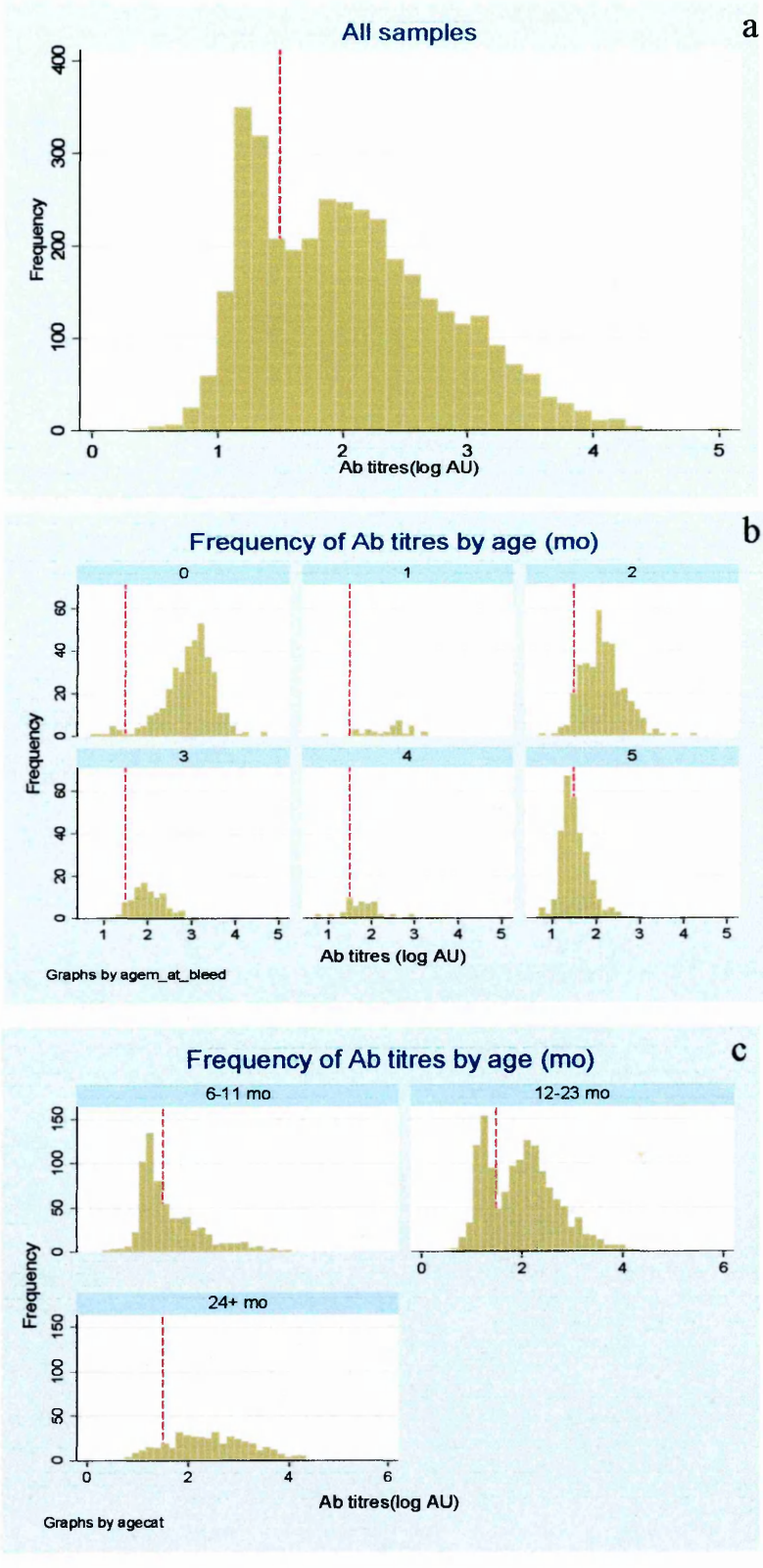


Figure 7.2. Frequency distribution of antibody titres (log AU) for the entire population (a) and for the population by age groups in months (b & c). Cut-off between seropositive and seronegative is shown by dashed red line at around 1.5 (log AU).

It should be noted that the number of samples in each month of age was very variable, but predominantly at cord, 2, 5 months etc because of the 3 monthly sampling regimen.

7.6.4 Duration of RSV seropositivity

The age seroprevalence profile for the whole population excluding known IFAT-CSR is depicted below in Figure 7.3a. At the cut-off level (1.495 log AU) for seropositivity, it was noted that the seronegative population comprised 28% of the total. At birth 97% of infants were born with RSV-specific matAbs, which appeared to gradually decline with time, eventually reaching a nadir of approximately 35% at 8-9 month. Thereafter, the proportion seropositive rose quite rapidly to 50% at 10-12 months, and to 92% by the time infants attained 31-36 months of age (Figure 7.3a).

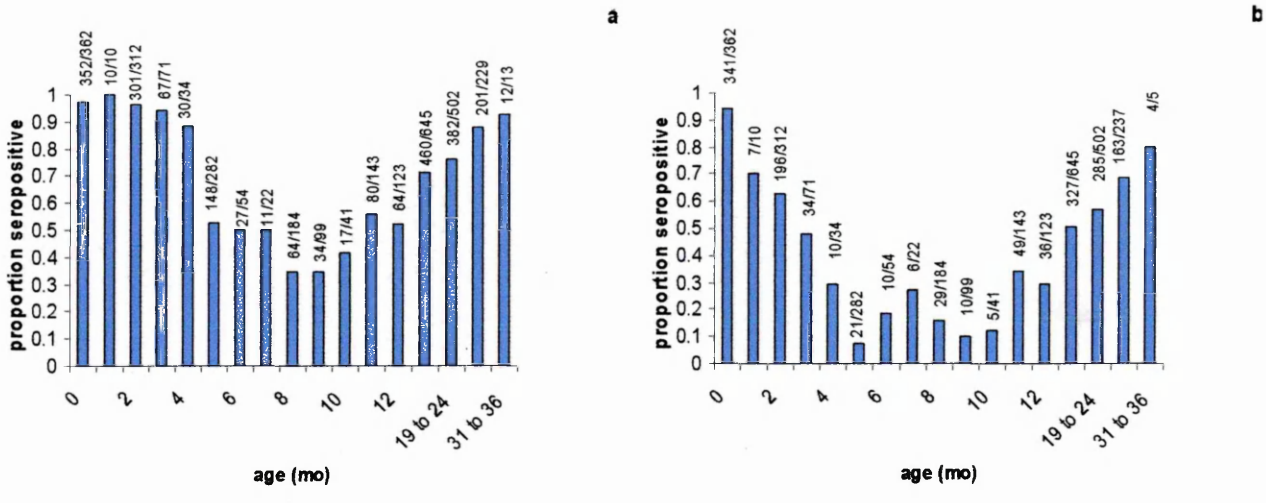


Figure 7.3. Age-seroprevalence profile (a) at assay cut-off level of seropositivity, 1.49 log AU and (b) at a cut-off of seropositivity of 2.0 log AU. Numbers indicated above each bar are number of children seropositive per total children in each age group.

If the level of seropositivity was raised to 2.0, where 50% of the population was defined as being seronegative, then the proportion of infants at birth with RSV matAbs dropped to 94%. By 5 months of life only 7% of children remained seropositive and a low average of

approximately 12% in ages 5- 10 months. Above 12 months of age, there was a steady increase in the proportion seropositive with age, presumably acquired from infection, with 70% in age group 25-35 months.

7.6.5 Characterization of RSV-specific antibody

7.6.5.1 Concentration of RSV-specific antibodies with age

The mean Ab titre for seropositive infants is depicted in Figure 7.4. It was noted that matAb declined over the first six months of life to a minimum mean Ab titre level of 62.85 (sd± 2.15) AU; thereafter, there was a rise in titres (acquired immunity) over time. From the scatter plot of Ab titres with age for the < 6 month old infants, it was seen that Ab titres for the majority of infants, Ab titres declined with time. As infants became older, an increase in Ab titres was observed, and by the time infants attained 24+ months, the majority had Ab titres above the cut-off level of seropositivity. RSV-specific Ab titres for all samples and by different age groups are shown in Figure 7.5 a-e.

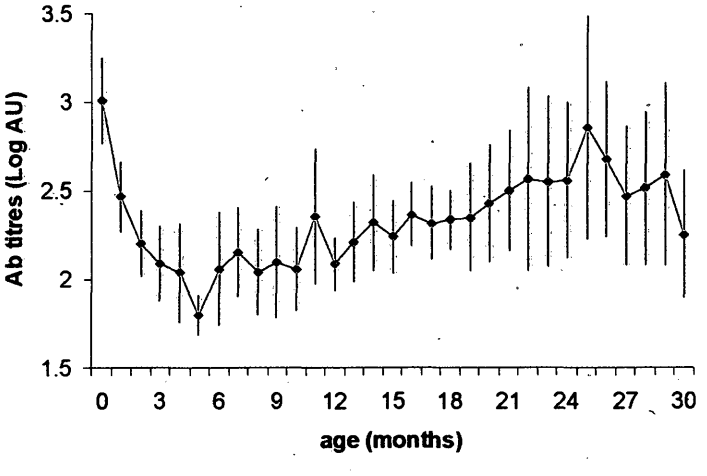


Figure 7.4. Mean antibody titres for all samples defined as seropositive by age. The vertical lines indicate the variance.

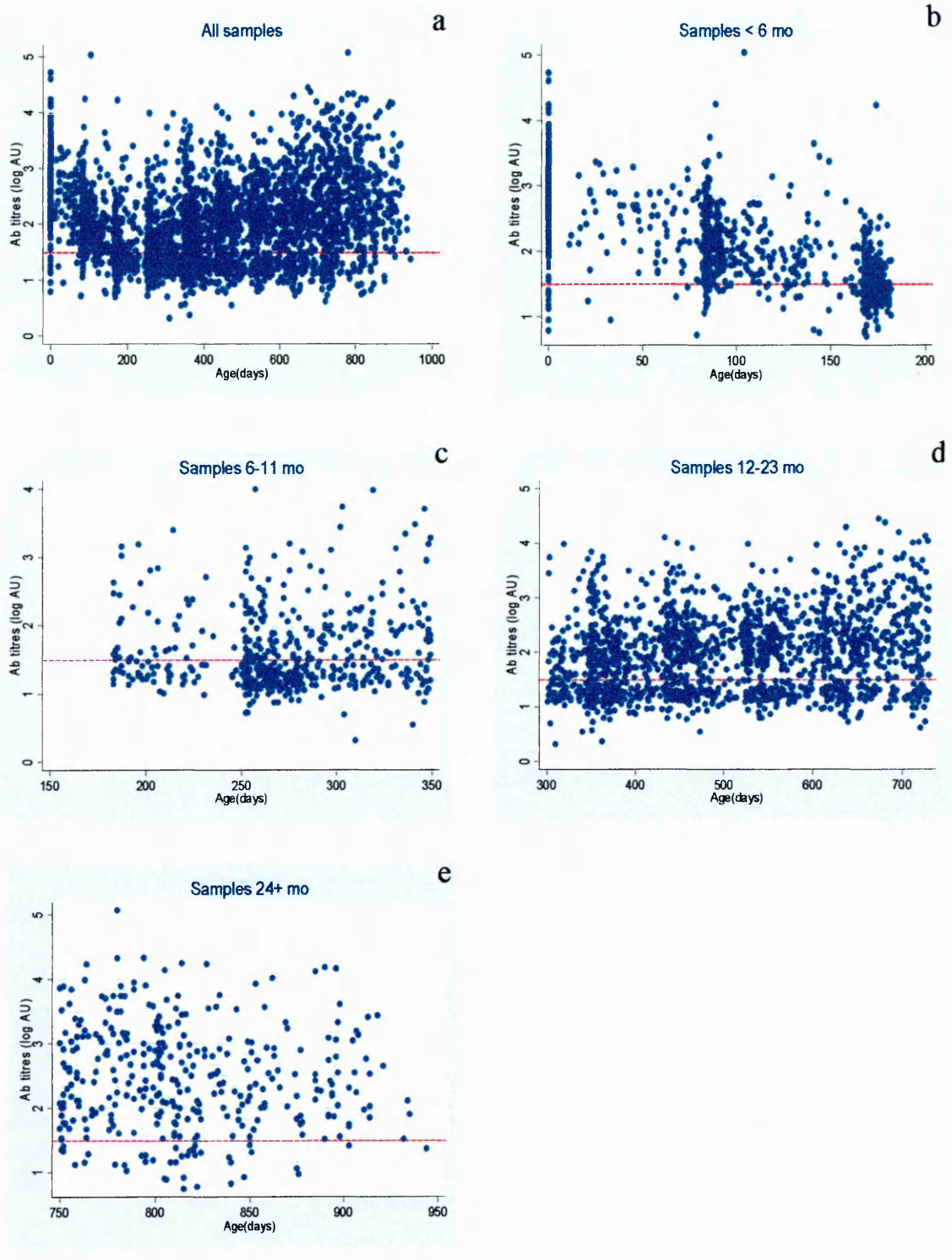


Figure 7.5. Scatter plots of RSV-specific antibody levels (log AU) for all samples (a) and by age group (b-e) for cohort children in Kilifi, Kenya. The cut-off for seropositivity is depicted by the dashed red line (log AU=1.495).

7.6.5.2 Analysis of the antibody response model

As the rate of decay of matAb was assumed to occur exponentially in this population, a linear regression model was used on all the data points for children less than 6 months of age irrespective of whether they experienced an IFAT- or ELISA-CSR (Figure 7.6) and the rate of decay calculated.

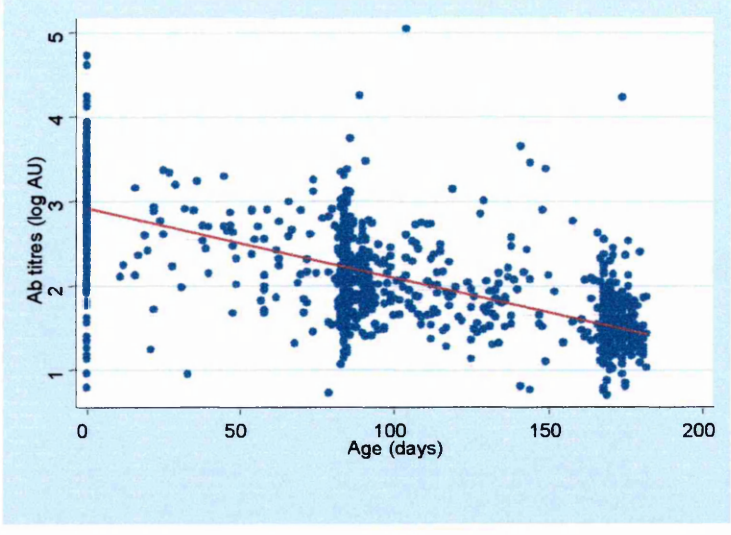


Figure 7.6. Scatter plots of Ab titres (log AU) during the first 6 months of life with predicted line of fit (maroon line) by simple linear regression (rate of decay (slope), m=-0.008 log AU/day; intercept, c=2.928 log AU)

To calculate the 'pristine' rate of decay in the seropositive population, infants who experienced at least 1 infection, whether IFAT-CSR or ELISA-CSR at both seroconversion levels, were excluded. This gave 2 population groups- non-infected and infected population (refer back to section 7.5) A random effects mixed model to account for the multiple measurements per child, was used, in which both the random effects (RE) and the fixed effects (FE) approach were compared to each other. No significant difference occurred between outcome measures by these two types of models, and hence the RE model was employed for all subsequent analysis.

The 'pristine' half life ($T_{1/2}$) in days of RSV-specific matAb for the seropositive population was 79 days (95% CI: 75.8, 81.4) whilst the mean duration (in days) of RSV-specific matAbs was 113 (95% CI: 109.3, 117.4) when infections were omitted at the 4-fold seroconversion level. These values were seen to be similar to the non-infected population in which infections at the 2-fold level were excluded and for the whole population group which was inclusive of infections at either seroconversion levels (Table 7.2).

Table 7.2. Linear regression model parameters, with estimated biological half life ($T_{1/2}$) and mean Ab duration for different population groups from a cohort of children less than 180 days of age in Kilifi, Kenya (see equations (4) & (5), section 3.3.1 for calculation of $T_{1/2}$ & mean Ab duration)

Population group (n) Model type	Model parameters	Estimates (slope, m)	Biological $T_{1/2}$ (95% CI) days	Mean duration (days) (95% CI)
All (1223) single level	Age (days) Adjusted R^2 Prob>F	-0.008285 0.5499 < 0.05	83.7 (79.6, 88.1)	120.7 (114.9, 127.2)
Non-infected (876) single level	Age (days) Adjusted R^2 Prob>F	-0.008433 0.5825 < 0.001	82.2 (77.8, 87.1)	118.6 (112.3, 125.6)
All (1223) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008257 0.5502 2138.67 <0.001	83.9 (80.5, 87.7)	121.1 (116.2, 126.5)
Non-infected (876) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.00841 0.5830 1822.40 <0.001	82.4 (78.8, 86.4)	118.9 (113.6, 124.6)
Non-infected popn {excluding all infants showing 2-fold seroconversion} (835) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008953 0.6595 3682.75 <0.001	77.4 (75.0, 80.0)	111.7 (108.2, 115.4)
Non-infected popn {excluding all infants showing 4-fold seroconversion} (851) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008787 0.6449 3115.75 <0.001	78.9 (76.2, 81.9)	113.8 (109.9, 117.9)

Seropositive (1025) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008057 0.4966 1586.09 <0.001	86 (82, 90.5)	124.1 (118.3, 131.5)
Seropositive: non-infected popn (743) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008449 0.5309 1506.38 <0.001	82 (78.1, 86.4)	118.4 (112.7, 124.7)
Seropositive: non-infected popn {excluding all infants showing 2-fold seroconversion} (693) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.009060 0.5811 2458.20 <0.001	76.5 (73.6, 79.7)	110.4 (106.2, 114.9)
Seropositive: non-infected popn {excluding all infants showing 4-fold seroconversion} (707) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008828 0.5692 2059.46 <0.001	78.5 (75.3, 82.1)	113.3 (108.6, 118.4)
Seropositive- levels <1.495 adjusted* (1223) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008306 0.5595 2215.20 <0.001	83.5 (80.1, 87.1)	120.4 (115.6, 125.6)
Seropositive- levels <1.495 adjusted*: non-infected popn (876) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008471 0.5896 1852.32 <0.001	81.8 (78.3, 85.7)	118.1 (112.9, 123.7)
Seropositive- levels <1.495 adjusted*: non-infected popn {excluding all infants showing 2-fold seroconversion}	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.009006 0.6635 3616.54 <0.001	77 (74.5, 79.6)	111 (107.5, 114.8)

(835) random effects				
Seropositive- levels <1.495 adjusted*: non-infected popn {excluding all infants showing 4-fold seroconversion} (851) random effects	Age (days) Overall R^2 Wald X^2 (df=1) Prob> X^2	-0.008834 0.6488 3064.33 <0.01	78.5 (75.8, 81.4)	113.2 (109.3, 117.4)

Popn indicates population; * indicates that for all infants with Ab levels <1.495 logAU, these titres were adjusted to 1.224 logAU (mean of seronegative population-see Appendix XIa). Shaded area is what is reported above and excludes infections at commonly reported seroconversion level of 4-fold or more.

A seropositive population comprises individuals with Ab titres $\leq$ 1.495 log AU (section 7.3)

7.6.6 Identification of outliers

7.6.6.1 Regression Diagnostics

Regression diagnostics allows for the verification that the data set meets the assumptions underlying linear regression. Additionally, this also allowed for the investigation of the effects of possible outliers, observations with large residuals, as well as and for their influence on the analysis (http://www.ats.ucla.edu/stat/stata/webbooks/reg/chapter2/statareg2.htm; Altman, 1991). An observation is said to be influential if by removing it from the regression model, it results in substantial changes in the estimate of coefficients. This is not a strict assumption of regression but such extreme values can have a significant effect on estimates of the model parameters.

A plot of residuals of predicted values against age (Figure 7.7a) revealed no pattern, and thus, the residual variance was homoscedastic (if there was variability in the variance, heteroscedasticity, the residuals would have been seen for example, to increase with age); while the studentized residuals identified one possible outlier (Appendix X). An outlier with leverage is defined as an observation with an extreme value on a predictor variable, therefore having undue influence (http://www.ats.ucla.edu/stat/stata/webbooks/reg/chapter2/statareg2.htm; Hamilton, 2006). A plot of leverage versus residual squared, as a quick means of visually checking for outliers with leverage and hence influence, was constructed (Figure 7.7b). This identified an outlier in addition to that identified by the studentized residuals. Omission of these possible outliers from the model had no effect on model parameters, and so these data points were retained in the model.

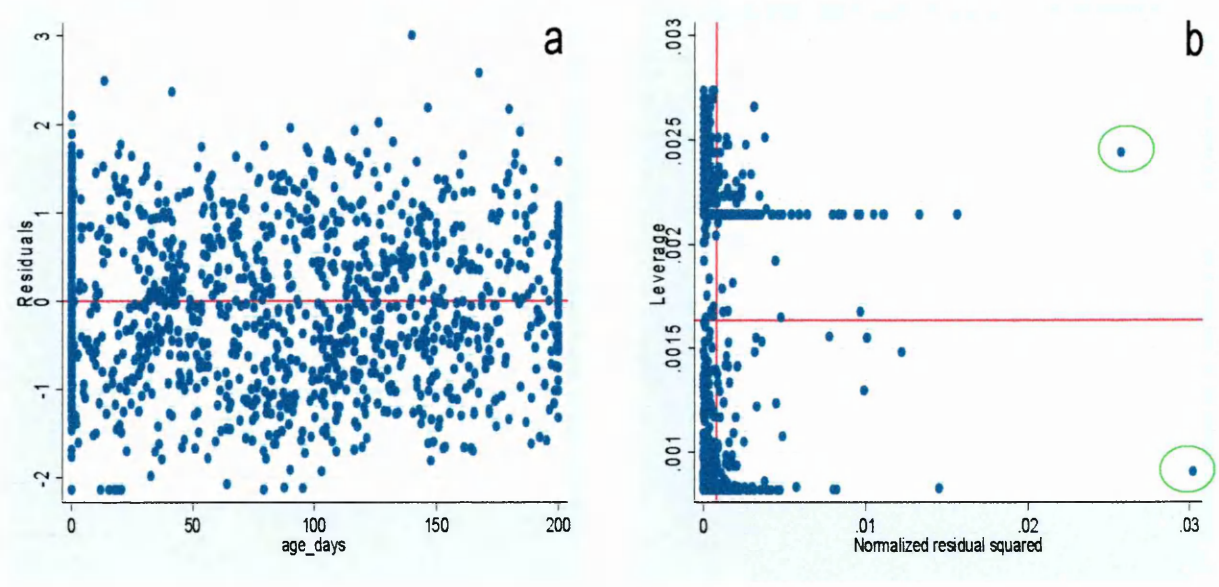


Figure 7.7. Regression diagnostic plots following log-linear regression on RSV-specific Ab levels in children ≤ 6 months. Where (a) residual-versus-predictor, age (in days) and (b) leverage-versus-residual-squared. The horizontal red line marks the means of leverage, and the vertical red line, the squared residuals. The green elliptical circles mark possible outliers. Note: this data set is of children ≤ 6 months, although scale is expanded to 200 days as clustered data was spread out in an attempt to look for possible patterns that violate regression assumptions

7.6.6.2 Distribution of gradients

Only children with 3 or more data points (which allowed for directional analysis) between 0-6 months were considered when testing for possible differences and associations with risk factors, between the half-life ($T_{1/2}$) of RSV-specific matAbs in the infected and non-infected population. A histogram plot of gradients (Appendix XIb) identified several outliers, which were thus omitted.

7.6.7 Student's t-test: possible differences in cord blood titres of non-infected and infected population's

Possible differences in cord blood levels of both infected and non-infected populations were evaluated using a two-sample *t-test* with unequal variances. Its association with risk factors was also evaluated at the two previously defined seroconversion levels.

Initially, a plot of cord blood levels by date of birth which distinguished between infants born in and out of epidemics (Figure 7.8) appeared to show no differences between cord levels in or out of an epidemic.

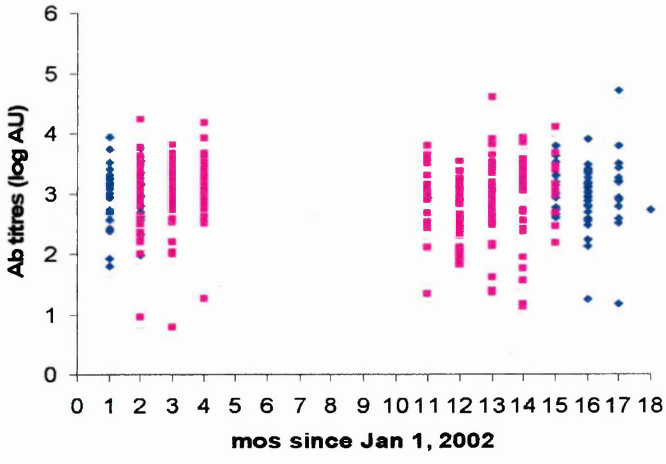


Figure 7.8. Cord blood levels in relation to date of birth, depicting children born in (N=242) ■ and out (N=119) ◆ of epidemics

In an attempt to tease out possible trends better between those born in or outside of an epidemic, a moving average of 7 days was investigated (Figure 7.9). In the 1st cohort there appeared to be no discernable trend, however in the 2nd cohort there appeared to be a slight increase in Ab titres as the epidemic season progresses and this was maintained for a while into the non-epidemic period whereupon a slight decline was noted with time.

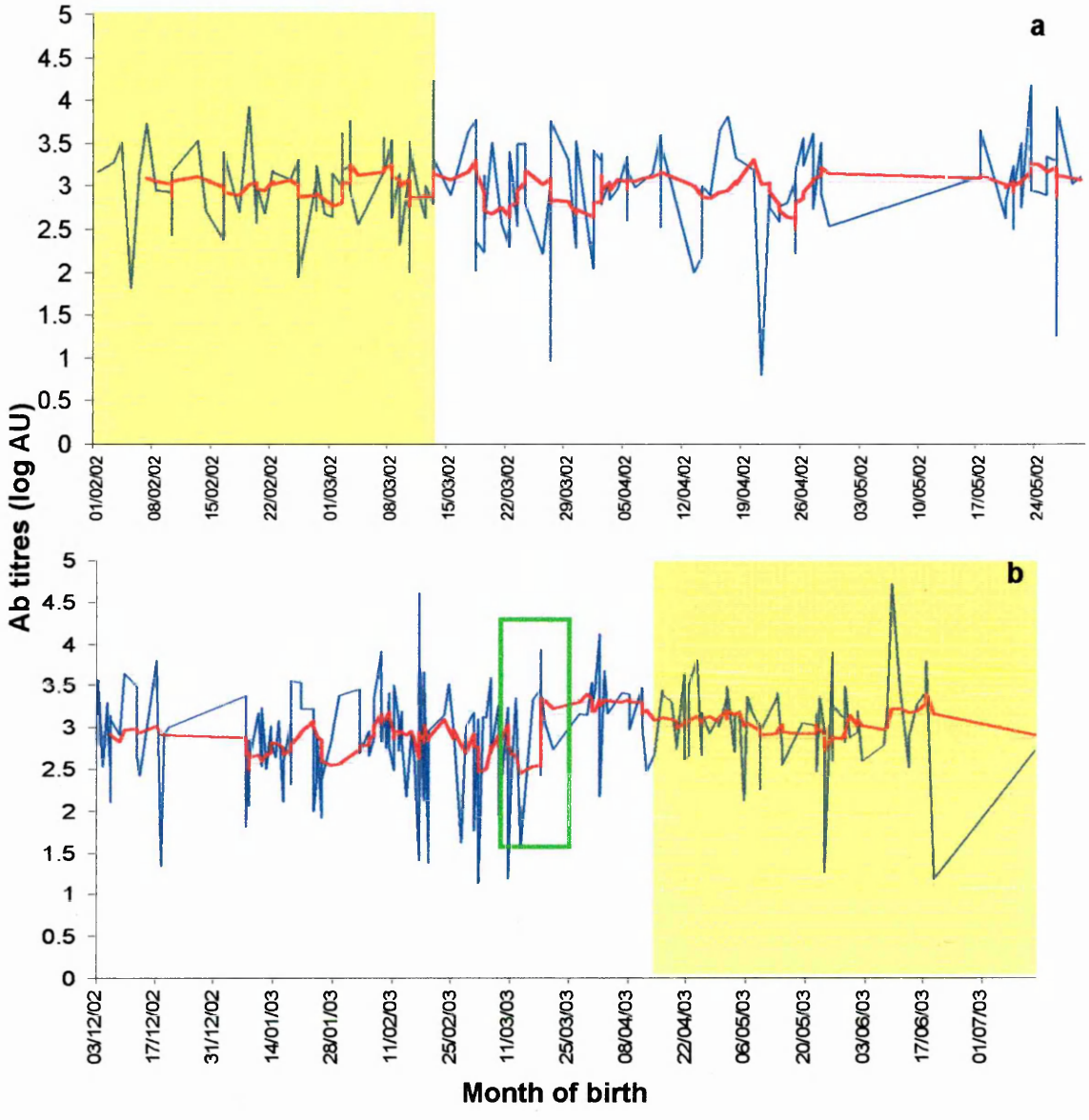


Figure 7.9. Cord blood levels in relation to date of birth (blue line) with moving average trend depicted (red line). Non-epidemic period depicted by shaded area. The (a) 1st and (b) 2nd three hundred children recruited to the birth cohort respectively. Green box shows switch in Ab titres from a lower to a higher level.

When infants were stratified into those who went on to experience an infection and those who did not within the 1st six months of life, there is evidence of a difference (*P=0.023*) in cord blood levels between these 2 groups using the 2-fold seroconversion level, but this evidence

diminishes ($P=0.09$) when the 4-fold cut off is used. Mean cord blood levels for the infected subgroup were seen to be lower in comparison to the non-infected subgroup (Table 7.3a & b).

Table 7.3a. Two-sample t-test with unequal variances of cord blood titres of infected & non-infected samples at 2-fold seroconversion level (Group 0 and 1 denote non-infected and infected populations respectively).

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     287    3.019845    .0297724     .504376    2.961245    3.078446
       1 |      74    2.810244    .0854398    .7349807    2.639962    2.980525
---------+--------------------------------------------------------------------
combined |     361     2.97688    .0297181    .5646434    2.918437    3.035323
---------+--------------------------------------------------------------------
    diff |            .2096018    .0904784                .0298896    .3893139
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   2.3166
Ho: diff = 0                     Satterthwaite's degrees of freedom =  91.4601

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9886         Pr(|T| > |t|) = 0.0228          Pr(T > t) = 0.0114
```

Table 7.3b. Two-sample t-test with unequal variances of cord blood titres of infected & non-infected samples at 4-fold seroconversion level (Group 0 and 1 denote non-infected and infected populations respectively).

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     293    3.006771    .0302666    .5180811    2.947202    3.066339
       1 |      68    2.848085    .0876619    .7228783    2.673111    3.023059
---------+--------------------------------------------------------------------
combined |     361     2.97688    .0297181    .5646434    2.918437    3.035323
---------+--------------------------------------------------------------------
    diff |            .1586856    .0927398               -.0257488     .34312
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   1.7111
Ho: diff = 0                     Satterthwaite's degrees of freedom =  83.6533

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9546         Pr(|T| > |t|) = 0.0908          Pr(T > t) = 0.0454
```

Furthermore, when birth in or out of an epidemic period was now taken into consideration, it was observed that there existed differences in matAb titres between the 2 population groups but only for children born within an epidemic. The matAb levels for the infected population

group was significantly lower than that for the non-infected group both at the 2-fold (*P=0.021*) and 4-fold (*P=0.048*) seroconversion levels (Table 7.4a & b; Appendix XIIb i-ii).

Table 7.4a. Two-sample t-test with unequal variances of cord blood titres of infected & non-infected samples at 2-fold seroconversion level of children born in an epidemic (Group 0 and 1 denote non-infected and infected populations respectively).

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     198    3.011512    .0371283    .5224414    2.938292    3.084732
       1 |      44    2.706714    .1227761    .8144046    2.459113    2.954316
---------+--------------------------------------------------------------------
combined |     242    2.956094    .0383185    .5960958    2.880612    3.031576
---------+--------------------------------------------------------------------
    diff |            .3047973    .1282672                .0473061    .5622885
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   2.3763
Ho: diff = 0                     Satterthwaite's degrees of freedom =  51.1309

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9894         Pr(|T| > |t|) = 0.0213          Pr(T > t) = 0.0106
```

Table 7.4b. Two-sample t-test with unequal variances of cord blood titres of infected & non-infected samples at 4-fold seroconversion level of children born in an epidemic (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     201    3.002002    .0373701     .529813    2.928312    3.075692
       1 |      41    2.731032    .1282952      .82149    2.471738    2.990326
---------+--------------------------------------------------------------------
combined |     242    2.956094    .0383185    .5960958    2.880612    3.031576
---------+--------------------------------------------------------------------
    diff |            .2709705     .133627                .0021488    .5397922
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   2.0278
Ho: diff = 0                     Satterthwaite's degrees of freedom =  47.0079

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9759         Pr(|T| > |t|) = 0.0483          Pr(T > t) = 0.0241
```

The infected infant population with a weight greater than 2.5 kg at birth were observed to have significantly (*P=0.037*) lower matAb levels than the non-infected population but only at the 2-fold seroconversion level (Table 7.5, Appendix XIIb iii-v)

Table 7.5. Two-sample t-test with unequal variances of cord blood titres of infected & non-infected samples at 2-fold seroconversion level of children with a birth weight level > 2.5 kg (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      208    3.047826    .0331142    .4775796    2.982542     3.11311
       1 |       59    2.849631    .0873015    .6705757    2.674878    3.024384
---------+--------------------------------------------------------------------
combined |      267     3.00403    .0325179    .5313462    2.940005    3.068055
---------+--------------------------------------------------------------------
    diff |            .1981949    .0933708                .012209    .3841808
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   2.1227
Ho: diff = 0                     Satterthwaite's degrees of freedom =  75.4524

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9815         Pr(|T| > |t|) = 0.0371          Pr(T > t) = 0.0185
```

7.6.8 Factors associated with cord blood titres

With regards to matAb titres after controlling for birth weight level, birth order levels and being born in or out of an epidemic, both individually and together, the matAb titres for the infected groups remained significantly lower when compared to the non-infected groups both at the 2- and 4-fold seroconversion cut-off levels (Table 7.6).

Table 7.6. Multiple regression models on cord blood levels showing model outcome of main effects.

Stratifying variable(s)	Coefficient of independent variable (matAbs infected- matAbs non-infected popns†)	p	Adjusted R^2
Birth weight levels			
2-fold*	-0.2181391	0.003	0.03
4-fold*	-0.166283	0.028	0.01
Born in epidemic			
2-fold*	-0.217065	0.003	0.02
4-fold*	-0.1647097	0.03	0.01
Birth order levels			
2-fold*	-0.2246853	0.002	0.02
4-fold*	-0.1753541	0.022	0.01
Born in epidemic & birth weight levels			
2-fold*	-0.2235207	0.002	0.03
4-fold*	-0.1705382	0.025	0.01

†popns means populations; *All variables were controlled for infection at the indicated seroconversion level (see Appendix XIIIb i-viii for more information)

7.6.9 Possible differences in rate of decay of matAbs of non-infected and infected populations

A two-sample *t-test* with unequal variances was used to test the rate of decay or differences in gradient of matAb decline in the 2 population subgroups (non-infected and infected-children with 3 or more data points), and when stratified by birth in or out of an epidemic period, no differences were identified (Appendix XIIa i-iv). However, when considering birth weight levels, the gradient of matAb was noted to be significantly steeper at the 2-fold ($P=0.014$) and 4-fold ($P=0.025$) for the infected population compared to the non-infected population group but only for infants < 2.5 kg (Table 7.7a & b; see Appendix XIIa v-vi). As both cord blood

levels and birth order were stratified to more than 2 levels, their effects were best examined using regression analysis (section 7.6.10).

Table 7.7a. Two-sample t-test with unequal variances of gradient of infected & non-infected samples at 2-fold seroconversion level of children with a birth weight level < 2.5 kg (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      79   -.0088541    .0004314    .0038341   -.0097129   -.0079953
       1 |      20    -.001896    .0025445    .0113792   -.0072216    .0034297
---------+--------------------------------------------------------------------
combined |      99   -.0074484    .0006719     .006685   -.0087817   -.0061151
---------+--------------------------------------------------------------------
    diff |           -.0069582    .0025808               -.0123398   -.0015765
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -2.6961
Ho: diff = 0                     Satterthwaite's degrees of freedom =  20.1038

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0069         Pr(|T| > |t|) = 0.0139          Pr(T > t) = 0.9931
```

Table 7.7b. Two-sample t-test with unequal variances of gradient of infected & non-infected samples at 4-fold seroconversion level of children with a birth weight level < 2.5 kg (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      80   -.0087126    .0004488    .0040144    -.009606   -.0078193
       1 |      19   -.0021255    .0026712    .0116434   -.0077374    .0034864
---------+--------------------------------------------------------------------
combined |      99   -.0074484    .0006719     .006685   -.0087817   -.0061151
---------+--------------------------------------------------------------------
    diff |           -.0065871    .0027086               -.0122558   -.0009185
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -2.4319
Ho: diff = 0                     Satterthwaite's degrees of freedom =  19.0273

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0125         Pr(|T| > |t|) = 0.0251          Pr(T > t) = 0.9875
```

7.6.10 Factors associated with the rate of decline of matAb titres over the 1st six months of life

As it was shown that there existed significant differences between the infected and non-infected population when taking into consideration some of the known risk factors, multiple regression analysis of the risk factors previously defined was carried out by means of the following model:

$$y = c + m_1x_1 + m_2x_2 + \ldots\ldots\ldots m_px_p$$

where y is the rate of decay; c is the intercept; m_i the age in days; and x_i are the explanatory or risk factor variables, from i=1,p *i.e.* cord blood levels, weight of child at birth *etc.* This allowed for the control of these potential risk factors and the selection of significant independent risk factors associated with decline of RSV-specific matAbs and matAb starting titres.

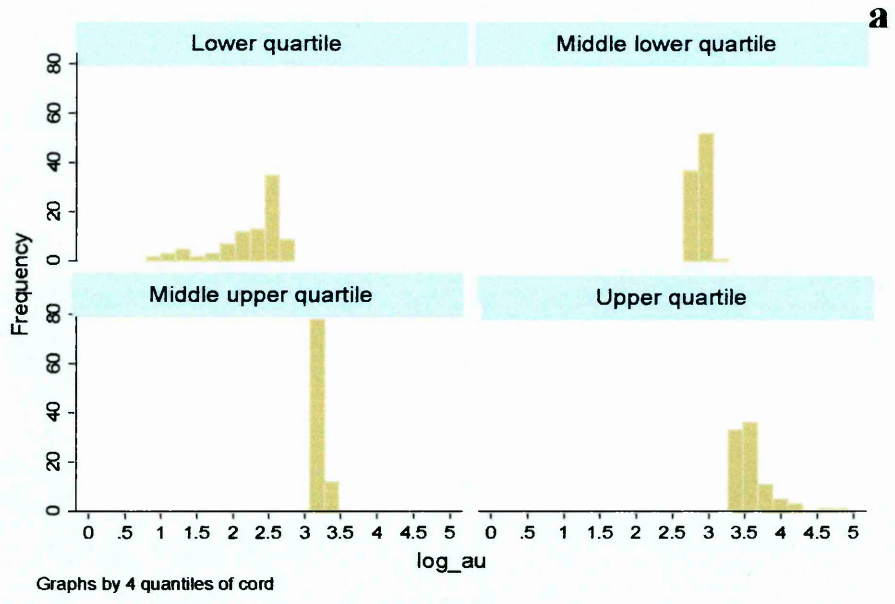
It was observed that none of the risk factors (Appendix XIIIa i-iii) except for cord blood levels were independently associated when the infected population was compared to the non-infected population (Table 7.8).

Table 7.8. Multiple regression models showing model outcome of cord blood levels controlling for all other risk factors at the both seroconversion levels

Stratifying variable	N	Coefficient of independent variable (age)	p	Adjusted R^2
Cord levels	181			
2-fold				
2		-0.0030211	< 0.001	0.43
3		-0.0043159	< 0.001	
4		-0.005967	< 0.001	
4-fold				0.43
2		-0.0030468	< 0.001	
3		-0.0043251	< 0.001	
4		-0.0059972	< 0.001	

Cord levels: 2, 3, 4 are middle lower, middle upper and upper quartiles respectively, cord level 1 omitted in regression analysis (see Appendix XIIIa iv-vii for more information).

Cord blood levels were divided into 4 quartiles (Figure 7.10a) with mean Ab titres being 2.24, 2.89, 3.19 and 3.60 log AU respectively. Therefore, between the 1st and 2nd, 2nd and 3rd and 3rd and 4th quartiles there was a 4.5-, 2- and 2.6-fold difference between Ab titres respectively. Figure 7.10b showed that the decay rate in each quartile were all constant and equal for each starting level of cord Ab.



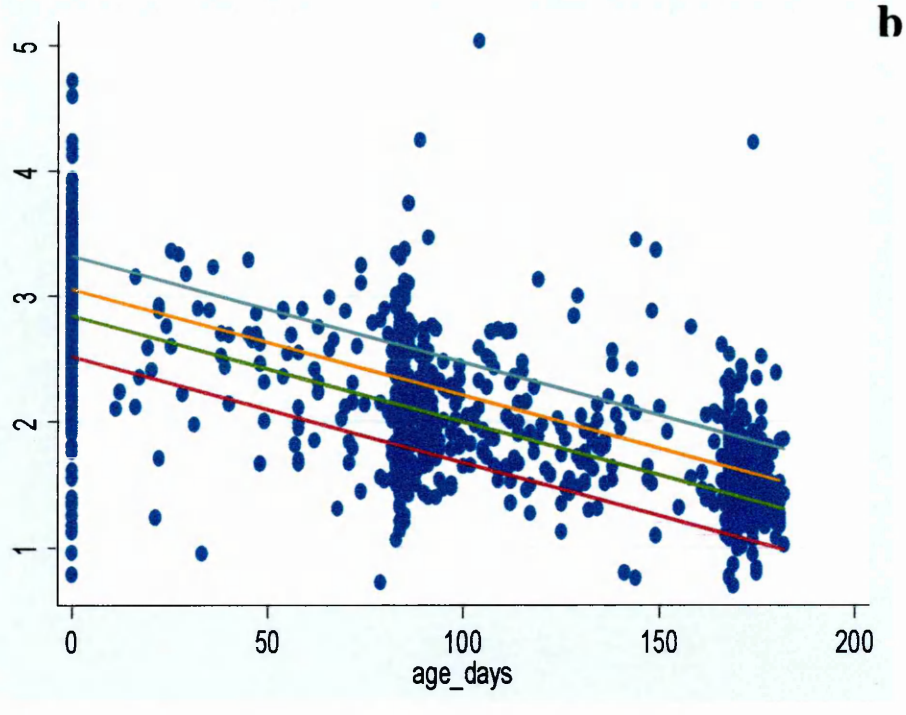


Figure 7.10. (a) Frequency of cord Ab levels by quartiles; and (b) predicted matAb decay rate in each quartile by multiple regression analysis. Predicted regression lines for lower quartile ——, middle lower quartile ——, middle upper quartile —— and upper quartile —— are shown.

7.7 Discussion

This study is unique in that there were a high number of study participants and the long follow-up time allowed for the extensive monitoring of RSV-specific serological changes from birth to approximately 3 years of age. The few studies that have been published all showed a decline in matAb levels but do not include 95% CI, as well, the population of infants below three years of age have been small, with the exception of Cox et al., (1998) who followed a total of 549 individuals, but with a very wide age range, 0-40 years. Additionally, Cox et al (1998) carried out a cross-sectional study design.

RSV infection was investigated in a birth cohort recruited in 2 phases between February – May 2002, and December 2002 – May 2003 respectively, from which a total of 361 cord bloods were collected. Cord blood titres and duration of RSV maternally-derived immunity, together with their association with risk factors were further explored. The decline of seropositivity with age was also examined.

Sera were screened quantitatively for total RSV-specific IgG using a simple ELISA technique, adapted from Wilson et al (2000). A cut-off point for seropositivity of 1.495 log AU was calculated from the ELISA and was further confirmed from the plot of frequency distribution for all sera samples (Figure 7.1 and Figure 7.2a). Maternal transfer was deemed to be efficient as approximately 97% of infants displayed RSV-specific matAb at birth. By 6-7 months, 50% infants were noted to be still seropositive (Figure 7.3a). This is in contrast to earlier studies (Table 3.3, chapter 3) with the exception of the study by (Ebihara et al., 2004), in which seroprevalence levels were seen to reach a nadir by 6 months of life, and seroprevalence varying from between 2-16% when carried out by the ELISA, and 21% by both the G- and F-

specific competition ELISA. In the study by Ebihara and colleagues (2004), a similar level of prevalence of 48% was observed. However, this was calculated for a wider age class that included 4-12 months old children, and furthermore, the numbers being reported are small (13/27). The authors do not offer an explanation for their results.

Varying the cut-off levels for seropositivity from approximately 1.5 (ascertained from assay) to 2.0 log AU (at this level, 50% of the population were considered seronegative), resulted in a quicker decline in seropositivity. A minimum Ab level was reached by 5 months. At this age, 7% of the population still remained seropositive. Thus, it would appear that on the basis of 50% of the population being seropositive, perhaps a childhood vaccine can be introduced soon after 3 months if it is assumed that matAbs have minimal interference when they get to levels below their $T_{1/2}$ levels. With regards to the cut-off level being set at 1.5, in which 72% of the population was classified as being seropositive, vaccine intervention would have to be delayed considerably to long after 6 months of age-but if you delay this long, you will fail to prevent many infections that you can see occurring in this age group. It still therefore remains important that the critical vaccination fraction (those fractions of each subpopulation that should be vaccinated to achieve protection against RSV) must be better established.

Subclinical or clinical infections as a result of repeat exposure to RSV could account for these high levels of seropositivity noted in this population, due to the boosting of matAbs (Cox et al., 1998). However, despite repeat attempts by PCR on 80 samples collected around the time point when a child was seen to seroconvert despite not showing any clinical symptoms (Section 6.4.6) we could not identify virus in these samples. Seroconversion in the absence of IFAT confirmed results will be discussed further in chapter 8. The study design, which thus

allowed for repeated analysis of a child, the frequent sampling frame and the total number of infants (503 infants) followed up, therefore allowed for a better estimate of seroprevalence with age in this population in comparison to previous studies, which may have underestimated the rate of conversion. Furthermore, an increase in seropositivity was noted especially above the age of 8-9 months in this population at the assay seropositive cut-off level and above 12 months when the cut-off level for seropositivity was raised to 2.0. This could be as a result of enhanced development in immune competence (Murphy et al., 1986a, Murphy et al., 1986b). However, this estimation of proportion seropositive is probably an underestimation of the average age at primary infection as most infants, especially under the age of 6 months do not seroconvert following first exposure (Cox et al., 1998).

Despite at least 50% infants being seropositive at 6-7 months age, using the cut-off value of 1.495 log AU, the rapid decline in RSV-specific Abs over the first 6 months of life is consistent with the loss of matAb as previously described (Cox et al., 1998; Brandenburg et al., 1997), as noted from the decline in Ab titres (Figure 7.5). In general, it is assumed that RSV matAb declines with age following an exponential pattern. Thus, we obtained a constant α, which described the rate of decay of RSV matAb that was independent of possible infection. This parameter was used to calculate the $T_{1/2}$.

The 'pristine' $T_{1/2}$ of RSV-specific matAb in the non-infected population, was estimated as 79 days (95% CI: 75.8, 81.4) and matAbs had an average duration of 113 days (95% CI: 109.3, 117.4). This $T_{1/2}$ is lower than that observed by Cox and others (1998), Hacimustafaoglu et al (2004) and Ward et al. (1983) who calculated $T_{1/2}$ that varied from between 91.2-100.3 (refer back to Table 3.2), although it is not clear as to whether the authors included or excluded

seronegative individuals with infections in their calculation of $T_{1/2}$, nor did they include 95% CIs. The $T_{1/2}$ for RSV-specific matAb however, was higher than that for measles, mumps and rubella (Caceres et al., 2000, Sato et al., 1979), and human parainfluenza type 3 (Lee et al., 2001), which vary from 35-40 and 51 days respectively, and hence RSV has a faster rate of decay in comparison.

Furthermore, the study suggests that the rate of matAb decay does not differ between the population that is inclusive of infections and the non-infected population. This was additionally confirmed following the 2-sample t-test (with unequal variance) on gradients of the infected and non-infected population. This therefore implies that RSV infections below 6 months of age have no effect on the rate of matAb decay. In other words, the presence of matAbs has a masking effect on infections under 6 months of life, and thus seroconversions in this age group will not be easily identified.

It is thought that cord blood levels can be influenced by various factors that include date of birth, being born in or out of an epidemic period (season) and birth weight (proxy for gestational age) as previously described (Glezen et al., 1981, Le Saux et al., 2003, Madhi et al., 2006). All cord sera were shown to be seropositive, with infants being born in or out of an epidemic seemingly displaying similar levels in this study. Although this was not clear from a plot of moving averages of cord titres of infants recruited in the 1st phase of the birth cohort, it appeared from those infants recruited in the 2nd phase, that with progressive time, cord titres appeared to increase and this was maintained partly into the non-epidemic period whereupon it was seen to decline slowly with time. It is assumed that children born later in an epidemic might have higher cord blood levels, because of the probability of increased risk of maternal

infection during pregnancy as the epidemic progresses. This observation is similar to earlier studies (Le Saux et al., 2003, Nandapalan et al., 1986) that reported an increase in RSV-specific Abs following an RSV epidemic. However the effect of unequal exposure time, or time-varying incidence, of infants to an epidemic period should be further explored.

The other risk factors affecting both RSV cord titres and RSV matAb decline were further analyzed using both the 2-sample t-test (with unequal variance) and multiple linear regression analysis. With respect to cord blood titres, children who went on to experience an infection within 6 months of age were observed to have consistently significantly lower titres in comparison to the children who did not experience any infection within this period. This difference was further maintained even when season was taken into consideration, both at the 2- and 4-fold seroconversion levels (Tables 7.5-7.6). This implies that matAbs are protective as earlier described (Glezen et al., 1981, Ogilvie et al., 1981, Roca et al., 2002). These authors observed that protection against RSV infection in early infancy was correlated with the level of matAbs. These observations therefore have implications for the development of a maternal vaccine (Hacimustafaoglu et al., 2004, Roca et al., 2002). Interestingly, infected infants with greater than 2.5 kg birth weight had significantly lower ($P=0.037$) matAb titres than those less than 2.5 kg, but only at the 2-fold seroconversion level. This is in contrast to knowledge that full term infants in comparison to premature have more efficient transfer of matAbs via the Brambell Fcγ receptors, thus have maternal IgG levels approaching adult levels at birth. This however is not replicated by preterm infants (de Sierra et al., 1993). The rate of matAb decline appeared to be significantly influenced by birth weight levels; the rate for the non-infected population appeared to decay at a much slower rate than the infected population following analysis by the t-test for infants less than 2.5 kg at birth. However, the use of t-tests

does not allow for the control of multiple covariates and hence multiple linear regression analysis was utilized.

On using this analysis method and having controlled for all other possible predictor variables, it was still observed that cord titres of the infected group remained significantly lower than the non-infected population (Table 7.8). Individually, these variables had no effect on the decay rate and their predictive power for the model, adjusted R^2, was extremely low. Inclusion of cord levels was able to explain between 41-43% of the model variation (Appendix XIIIa). However, it still remains important to calculate what the correctly specified model would be while taking into consideration the rate of RSV-specific matAb decay in this population in order to correctly assess vaccine impact in this population, in the presence of matAbs. The model makes the undesirable assumption that the immune system is a one compartment model, in other words, RSV matAb decay is only dependent on the inherent characteristics of these circulating Abs. Future studies should thus address a multi-compartmental model to better understand the constraints within which the immune system functions. However, as it stands, this model importantly demonstrates that the rate of decay in infants in Kilifi is 79 (95% CI: 75.8, 81.4) days.

The relatively short half-life of RSV matAb of about 2.5 months implies that a childhood vaccine could be administered fairly soon after this. However, 88% of the population is still seropositive by 4 months, and it is plausible that existing Ab titres could interfere with vaccine response and hence childhood vaccines may not be useful in this setting. Alternatively, as high levels of matAbs are protective, maternal vaccination might be a better alternative in this population. Apart from reducing the potential risk of infection to the mother and hence the

child, a maternal vaccine would augment matAb levels, which in turn would be transferred to the infant via transplacental transfer or through breast-feeding, protecting the infant during the vulnerable period of life when their immune system is still under-developed. Despite this, it still remains a public health issue as to what the recommended age for vaccination should be, especially within the developing country setting where a high proportion of the population is seen to still be seropositive by 6-7 months of age.

CHAPTER EIGHT

The dynamics of RSV infection within the Kilifi birth cohort: characteristics of the RSV IgG specific humoral responses

8.1 Introduction

The pathogenesis of RSV infection has not been fully elucidated and as thus, the immunological responses to RSV infection remain of immense interest. The role of Abs against RSV culminating in protective immunity (correlates of immunity) was previously reviewed (Section 3.5). Although precise correlates of immunity have not been ascertained (section 3.5), high titres of neutralizing Ab have been associated with protection against disease (section 3.4).

The serum samples collected in this study allowed for the investigation of the dynamics of infection within a rural population of Kilifi, Kenya thus allowing for the pattern of acquisition of RSV-specific IgG in the general population to be elucidated further. Two types of infections were observed during follow-up, those that were clinically confirmed in the laboratory by IFAT (IFAT-CSR), and those that were detected by ELISA only (ELISA-CSR), but had no corresponding IFAT result (see definitions, section 7.3). Previously, it was shown that children who had lower titres of matAb were more likely to go on to be infected (higher risk of incidence) compared with children with higher titres of matAb (section 7.6.9). Therefore, the effect of cord blood levels, in addition to other factors that included the number of previous infections experienced, the effect of previous titres, being born in or out of an epidemic period, on the degree of the Ab response were investigated. Due to the staggered

recruitment of the birth cohort, survival analysis was carried out, together with all other data analysis in Stata 9.0™.

8.2 Objectives

The objectives of this chapter therefore include the following:

- the description of the dynamics of the Ab response following natural RSV infection;
- the investigation into the role of cord blood levels of anti-RSV Ab in relation to disease outcome;
- the calculation of the average age of infection stratified by infection type, *i.e.* 1°, 2° *etc*;
- the effect of various factors on ensuing incident of infections (both ELISA-CSR and IFAT-CSR); and
- the establishment of a protective threshold

8.3 The analysis procedure

This involved the following steps:

(1) Exploration of RSV-IgG responses, serological responses of paired sera samples, and exploration of the difference between convalescent and acute samples in different age groups.

(2) Examination of individual acute/convalescent responses by infection type, 1^{st},2^{nd} *etc* in different age groups.

(3) Examination of the proportion of children showing seroconversion with age.

(4) Exploration of the effect of cord blood levels on paired samples and on disease outcome.

(5) Estimation of the protective threshold against RSV-associated LRTI.

(6) Examination of the incidence rates of IFAT-CSR and ELISA-CSR and effect of various factors, including cord blood levels, age at infection (< 6, 6-11, 12-23 and 24+ months), number of previous infections and being born in or out of an epidemic, on these rates.

8.4 Methods/Statistical Analysis

8.4.1 Survival Analysis

This was performed by the Kaplan-Meier method using Stata™ 9.0 in order to calculate incidence rates of infection for the study period. Date of recruitment was considered date of entry into the study, whilst date of diagnosis was used to define the outcome date. The data were thus evaluated for effects of age and infection type, having taken into account the staggered recruitment of the birth cohort. Time-dependent exposure to RSV was taken into account by assuming analysis time was calender time. The relative hazard of various factors likely to affect the incident rate of both ELISA-CSR and IFAT-CSR were further investigated using the exponential regression model.

Any difference seen between acute/convalescent Ab titres was quantified by either the Student's 2-tailed *t*-test with unequal variance, or ANOVA.

8.5 Results

8.5.1 RSV-specific IgG responses

On an individual basis, RSV IgG responses were seen to show considerable variation between infants. In some instances as noted for infants in Figure 8.1a, there was serological identification of cases undetected by surveillance (ELISA-CSR). The temporal pattern of Ab

responses for these children showed a decline of matAb titres to a minimum by 6-7 months. The minimal level reached varied, and it was noted in some instances that the level was below the cut-off of seropositivity *e.g.* for infants 3835 and 3920. Above 12 months, Ab responses remained elevated *e.g.* for infants 3830, 3835, rose gradually then declined as for infant 3920, or declined steeply as for infant 4247 post-infection. Also observed was the fact that for some children, *e.g.* infant 3920, no infection was noted before 18 mo despite them experiencing at least 2 epidemics. With respect to infants in Figure 8.1b some children were seen to experience 1 IFAT confirmed infection, during the 4 year follow-up period. Furthermore, as similarly observed for infants in Figure 8.1a, matAb titres declined to a minimum by 6-7 months, the minimum being observed to be below the cut-off of seropositivity for some infants, 3885 and 3748. Under 6 months of age, the ELISA was not able to record rising titres between acute/convalescent pairs for IFAT positive cases, such as in infants 4097 and 3748 (Figure 8.1b). However, above 6 months of age following examination of paired samples, rising titres were recorded *e.g.* in infants 3938 and 3834. Post-infection dynamics revealed that either Ab titres remained elevated (infants 3885 and 3938) or quickly declined (infants 4097 and 3748).

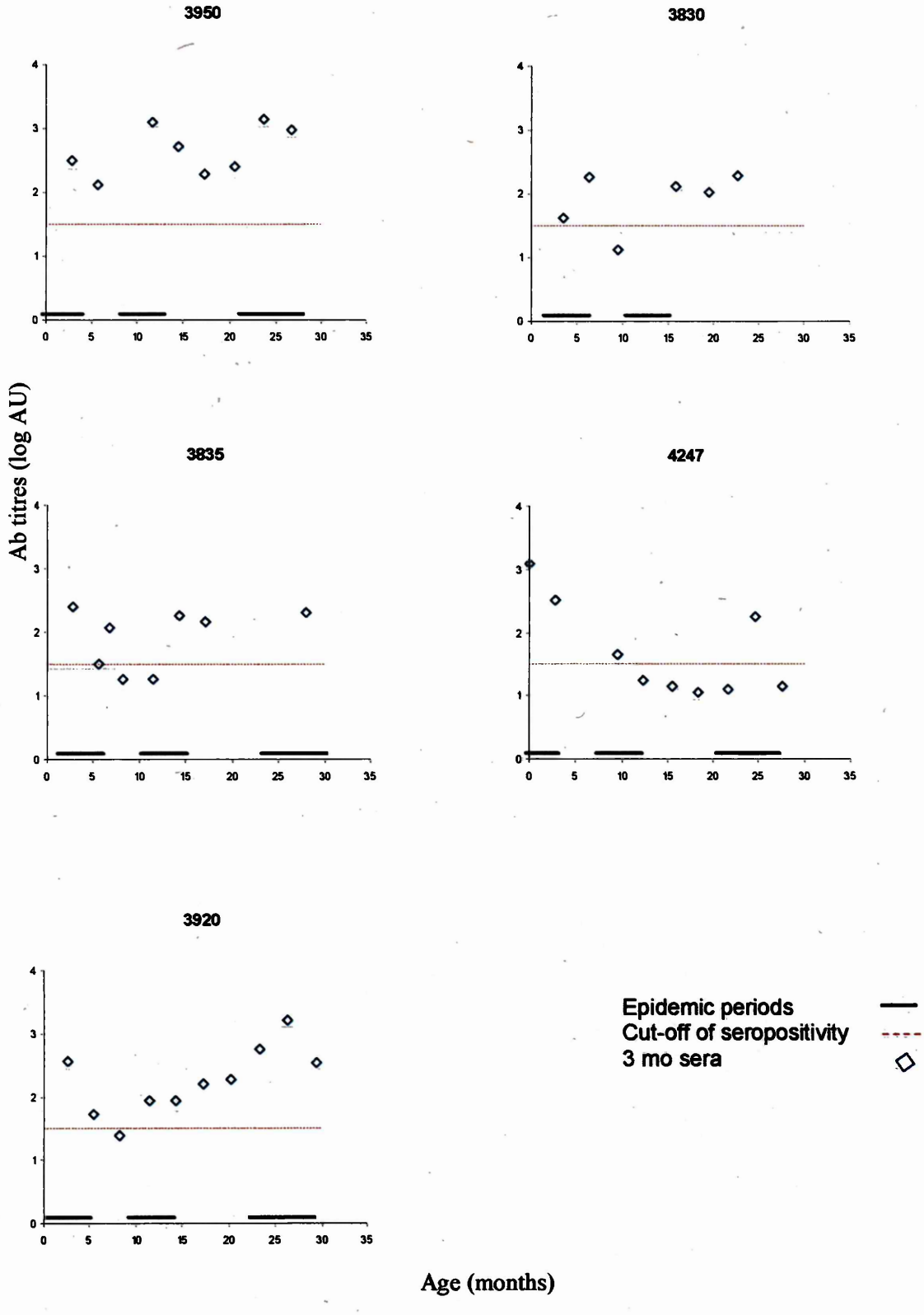


Figure 8.1a. Serological responses for a selection of children who showed no clinically confirmed infections (IFAT negative) over the follow-up period of the study.

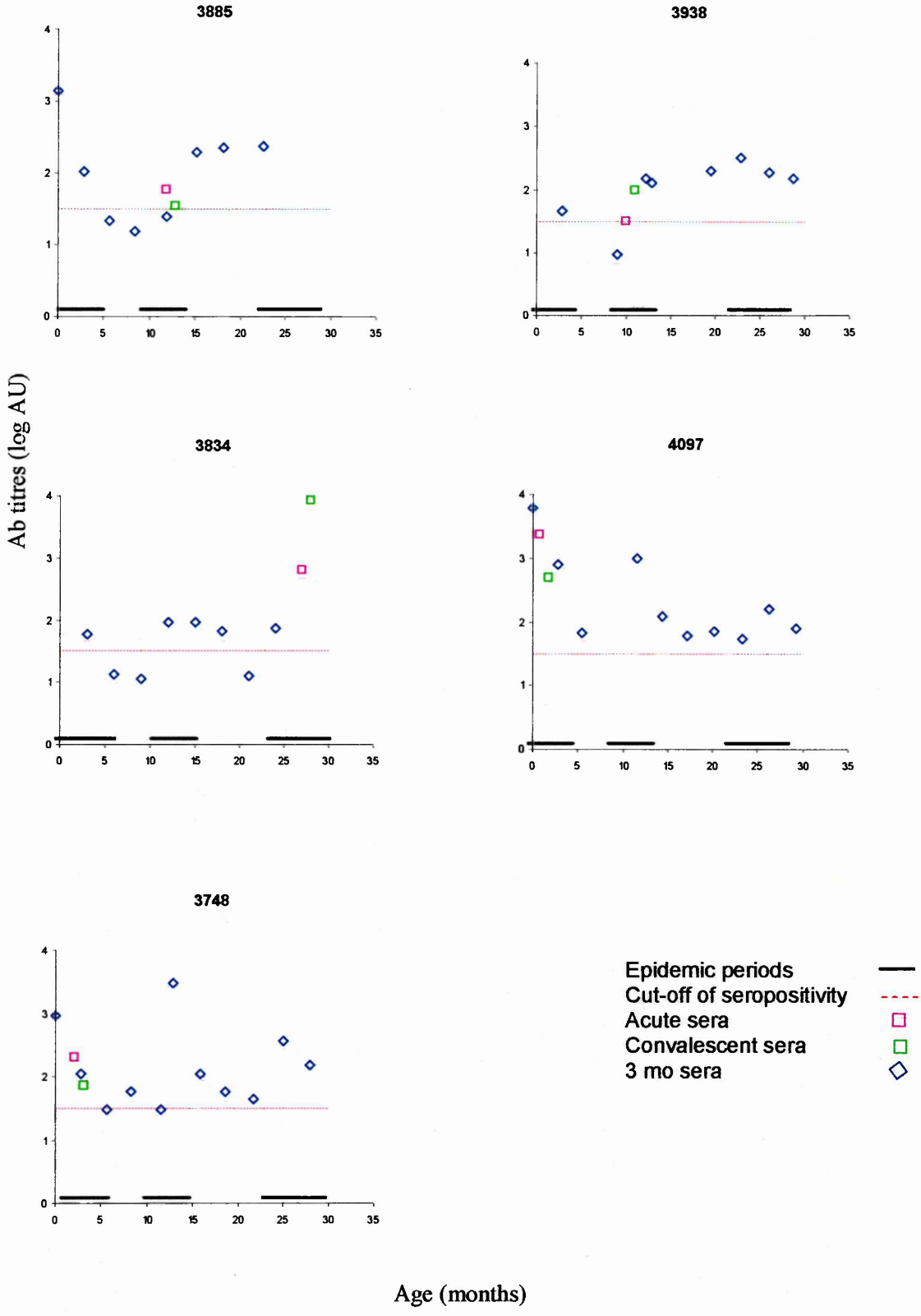


Figure 8.1b. Serological responses for a selection of children in whom only 1 clinically confirmed infection (IFAT positive) was recorded in the follow-up period of the study.

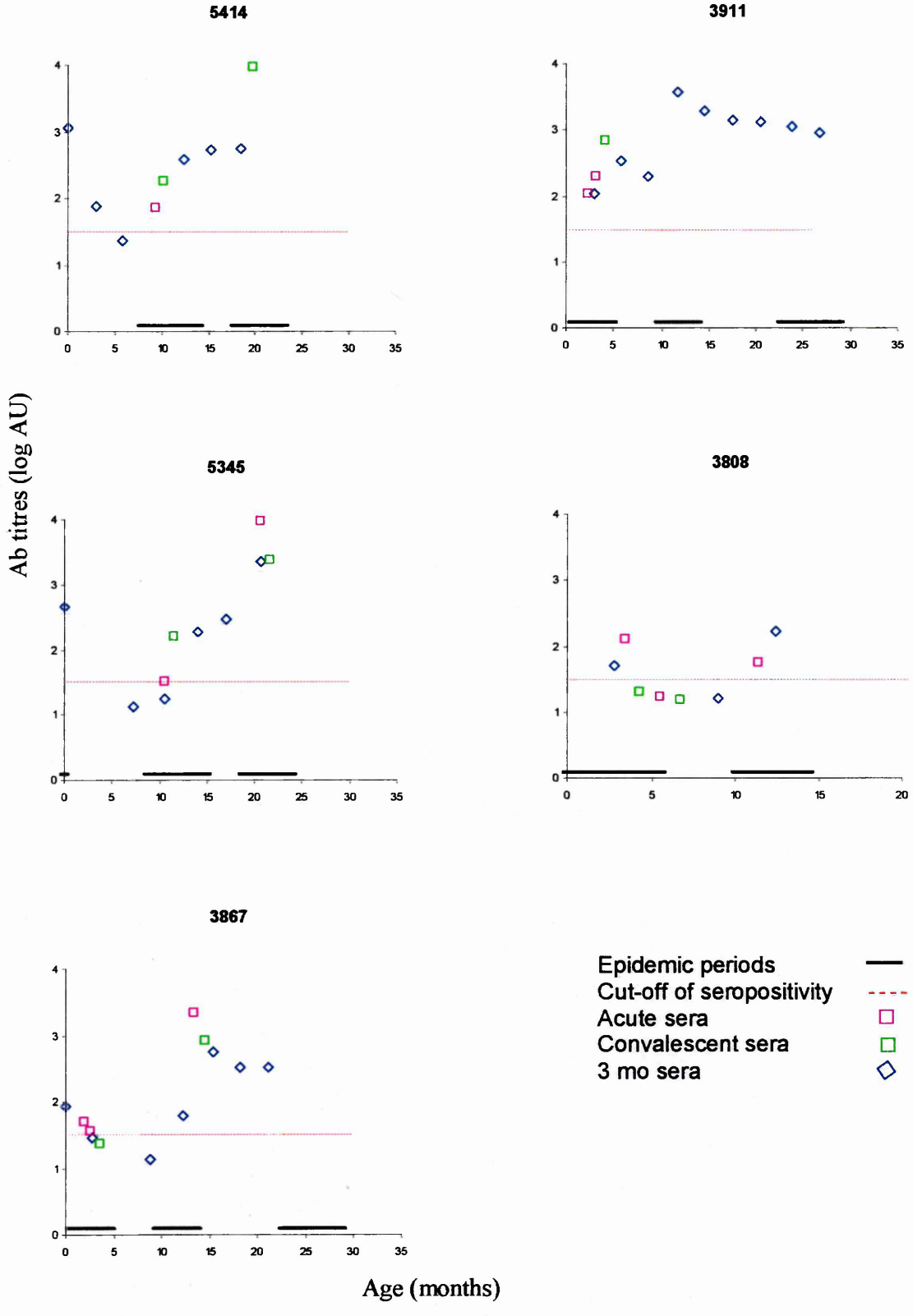


Figure 8.1c. Serological responses for a selection of children in whom > 1clinically confirmed infection (IFAT positive) was recorded in the follow-up period of the study.

Infants in Figure 8.1c showed similar Ab response kinetics as for infants in Figure 8.1b. However, these infants were observed to experience > 1 IFAT confirmed infection, during the follow-up period. Still the ELISA failed to record rising titres between acute/convalescent pairs for IFAT positive cases under 6 months of age as for infant 3867. Occasionally though, seroconversion was noted between acute/convalescent pairs under 6 months as for infant 3911. Again above 6 months of age, rising titres were recorded *e.g.* in infants 5345, with some infants, 5414 and 5345 showing classical boosting. It was observed that post-infection, Ab titres quickly declined (infants 5345 and 3867) or gradually declined (infant 3911).

8.5.2 Prevalence of RSV-specific Abs

Following an IFAT positive result, the presence of RSV-specific Abs was tested in paired samples by ELISA, acute samples being collected approximately 5- 7 days, and convalescent samples 3-6 weeks, post-infection. Of the total number of acute sera collected, 62.7% (185/295) were seen to be primary infections. With regards to infants experiencing 2, 3 and > 3 infections, 26.8% (79/295), 7.1% (21/295) and 3.4% (10/295) IFAT confirmed infections respectively were noted (Table 8.1).

Additional serological responses also occurred in the absence of IFAT confirmation (ELISA-CSR), although it should be noted that these occurred over a 3-month period rather than the 1-month period as between acute/convalescent pairs. Four hundred and ninety-nine (18%) missed infections or ELISA-CSR at the 2-fold seroconversion level were identified. With regards to ELISA at the 4-fold seroconversion level, an additional 317 (12%) missed infections were picked up by the assay. From the ELISA, it was noted that by 12 mo of age,

54% of infants had experienced their first infection, whilst by 24 mo of age 85% and 48% of infants had suffered 1 and 2 RSV infections respectively.

Table 8.1. The frequency of paired sera samples collected following confirmation of positive IFAT results during entire follow up period (N=281)

Sample type	Whole population (N=281) Frequency/percent	Under 6 mo population (N=94) Frequency/percent
Acute 1	185 (32.74)	57 (37.01)
Acute 2	79 (13.98)	24 (15.58)
Acute 3	21 (3.72)	5 (3.25)
Acute 4	7 (1.24)	1 (0.65)
Acute 5	2 (0.35)	-
Acute 6	1 (0.18)	-
Convalescent 1	165 (29.20)	50 (32.47)
Convalescent 2	76 (13.45)	15 (9.74)
Convalescent 3	21 (3.72)	2 (1.30)
Convalescent 4	7 (1.24)	-
Convalescent 5	1 (0.18)	-
Total	565	154

Note: Of these, there were 97, 39, 13 and 5 paired primary, secondary, tertiary and quaternary sera samples respectively.

8.5.3 RSV-specific Ab responses to clinically confirmed RSV infections

RSV-specific Ab responses between acute and convalescent specimen from different age groups were explored. The difference between mean Ab titres of convalescent and acute sera samples in different age categories is shown below (Figure 8.2).

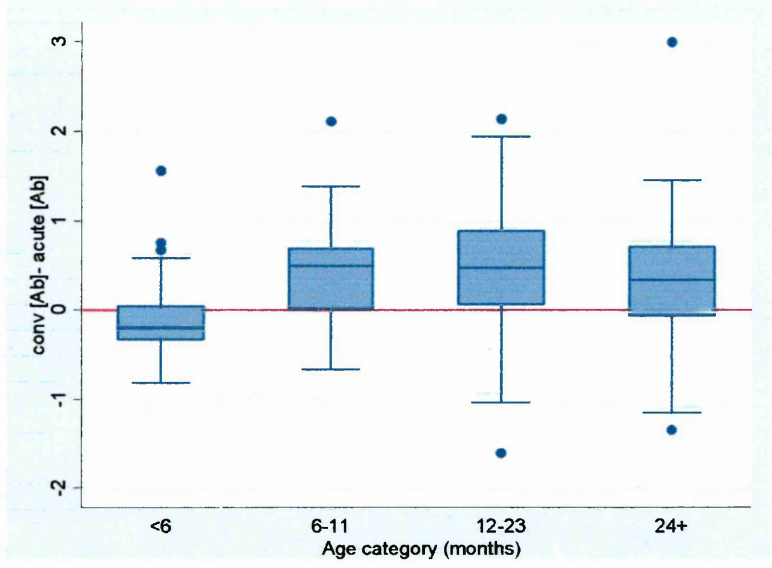


Figure 8.2. Mean differences between acute and convalescent titres by age category. [Ab] denotes Ab titres

Below 6 months of age, the mean difference was seen to be negative, and above 6 months these differences were positive, *i.e.* convalescent titres were higher than acute titres. The mean differences were only significant between paired sera in the < 6 month age group when compared to all other age groups, 6-11, 12-23 and 24+ months, in turn (2 tail *t*-statistics, *P=0.008*, *0.001* and *0.03* respectively).

With respect to serological responses in individually paired samples (Figure 8.3), it was observed that below 6 months of age, for the majority of infants, the acute titres were higher than the convalescent titres, only 12.5 % (4/32) of infants showed a 2-fold RSV-specific seroconversion. Additionally, there appeared to be a difference in average acute titres of the

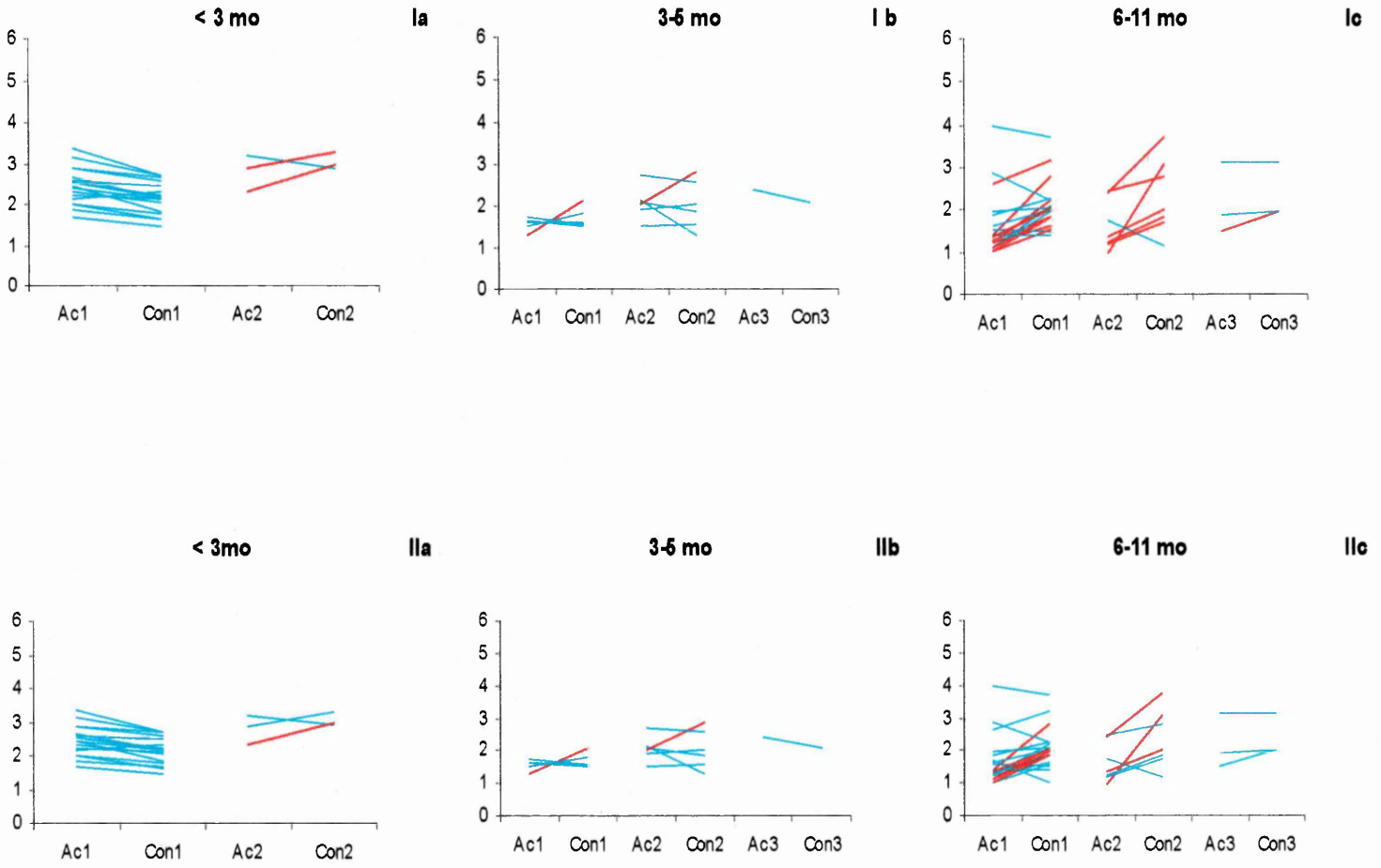


Figure 8.3. Serological responses between paired samples in different age classes, (a) < 3; (b) 3-5; (c) 6-11; (d) 12-23 and (e) 24+ mos. Panel I and II indicates 2-fold and 4-fold cut-off levels, respectively. Red lines depict serocoversions, turquoise lines depict no seroconversion at the 2 cut-offs between acute and convalescent specimens. Ac: acute; con: convalescent pairs, 1-4: primary, secondary, tertiary and quaternary infections.

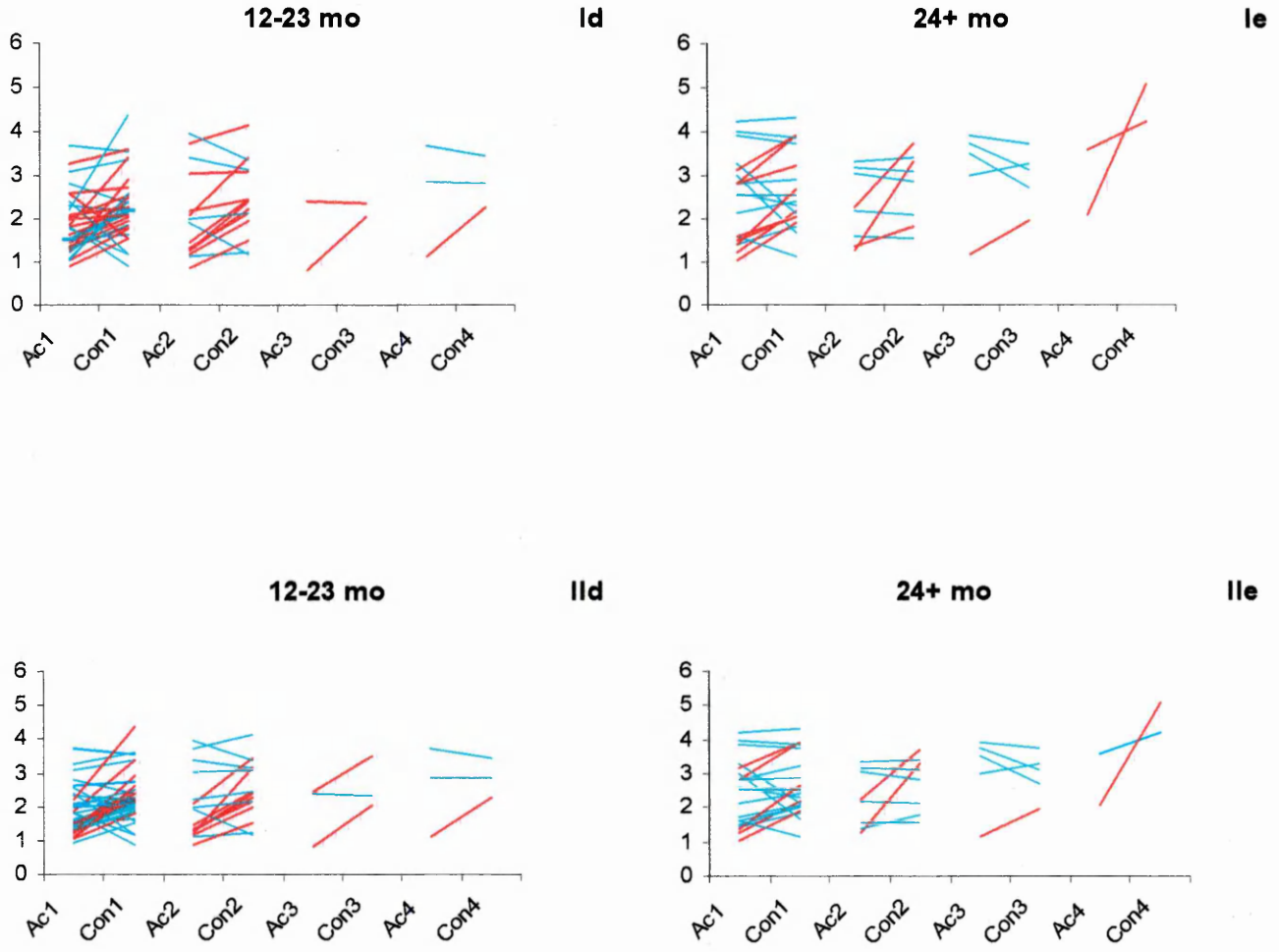


Figure 8.3 cont. Serological responses between paired samples in different age classes, (a) < 3; (b) 3-5; (c) 6-11; (d) 12-23 and (e) 24+ mos. Panel I and II indicates 2-fold and 4-fold cut-off levels, respectively. Red lines depict serocoversions, turquoise lines depict no seroconversion at the 2 cut-offs between acute and convalescent specimens. Ac: acute; con: convalescent pairs, 1-4: primary, secondary, tertiary and quaternary infections.

< 3 month old group as compared with the 3-5 month old group. Those of the former group were much higher in comparison to the latter group, probably reflecting the decay in matAbs. As children got older, the reverse was noted. More children's acute titres were lower or at the same level as their subsequent convalescent sera, and it was observed that with each repeat infection, mean convalescent titres appeared to increase towards cord levels (Figure 8.4).

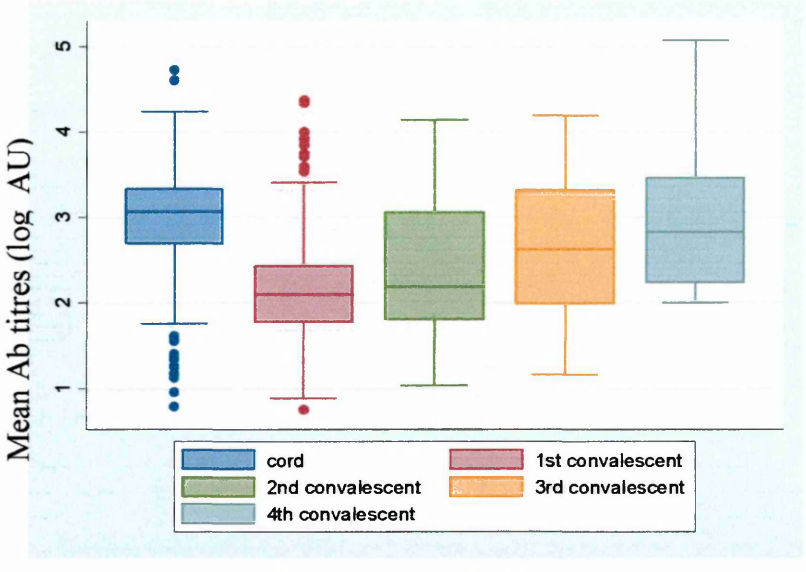


Figure 8.4. Mean convalescent titres following consecutive infections compared to cord blood titres

However, there existed a small proportion of children, about 21%, for whom acute titres still remained more elevated than convalescent titres. A similar trend was noted at the 4-fold seroconversion level.

The proportion of children exhibiting IFAT-CSR at the 2-fold seroconversion level is shown in Figure 8.5; a similar profile was noted at the 4-fold level. It was observed that above 8 months of life, there was a marked increase in the rate of seroconversion in comparison to the under 8 month olds.

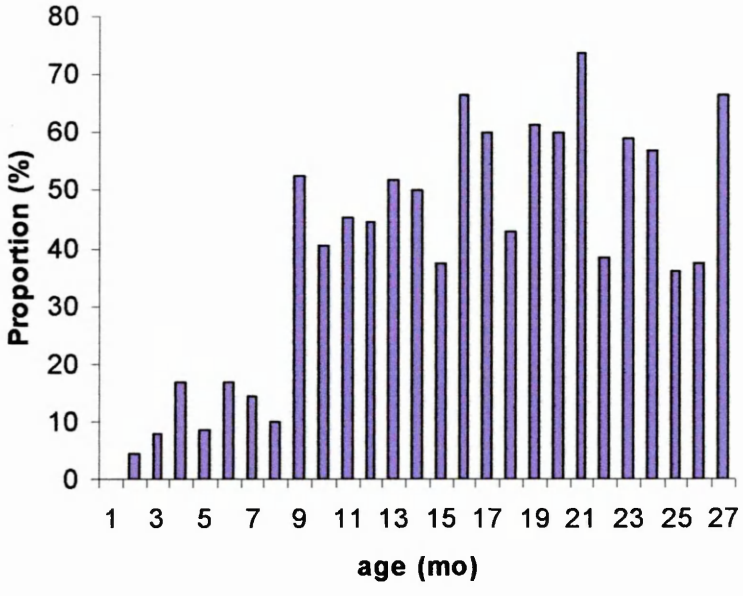


Figure 8.5. Proportion of children with IFAT positively confirmed infection showing 2-fold seroconversion with age.

8.5.4 The effect of cord blood levels on serological responses of paired samples

When the effect of cord blood levels on mean changes of Ab titres between paired samples were further investigated, Ab titre differences were noted to be either zero or positive (Figure 8.6a). When age was taken into account, below 6 months, irrespective of cord level, differences between paired sera were seen to be negative or nearly zero. There also appeared to be a decline in mean differences between the 2nd and 4th quartiles. Above 6 months of age, mean differences were observed to be positive. In the 6-11 month age class, there appeared to be a rise in mean difference from the 1st to the 4th quartile level, however, from 12+ months, no trend was noted, cord levels appeared to have no effect on serological response between acute and convalescent pairs (Figure 8.6b).

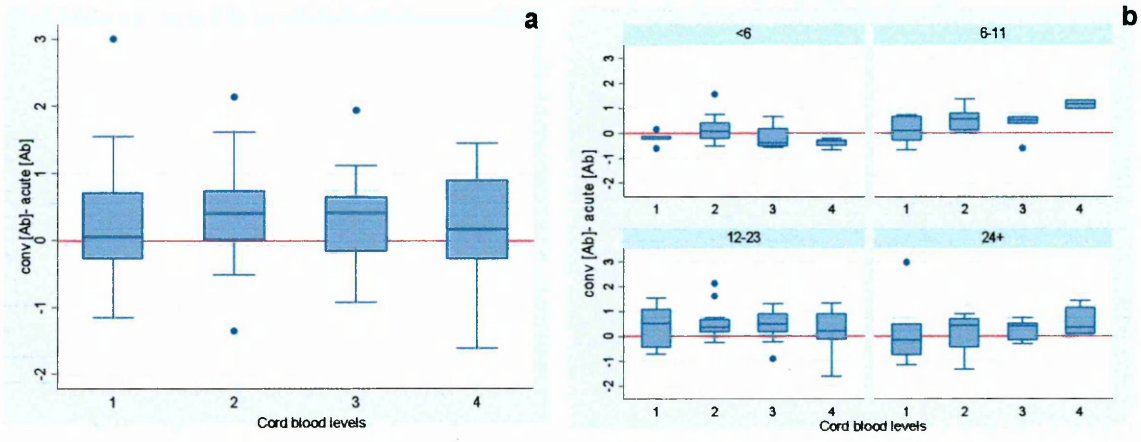


Figure 8.6. Mean differences between acute and convalescent paired samples. Mean differences are stratified by (a) cord blood levels, and (b) cord blood levels and age groups. [Ab] denotes Ab titres, 1,2,3,4 represent the lower (mean Ab titres=2.24 log AU), middle lower (mean Ab titres= 2.89 log AU), middle upper (mean Ab titres=3.19 log AU) and upper (mean Ab titres=3.60 log AU) quartiles, respectively.

8.5.5 The role of cord blood levels with disease outcome

Approximately 15% of pneumonias identified were due to RSV infection (Table 8.2).

Table 8.2. The number of pneumonia episodes in the cohort and those that were due to RSV (IFAT confirmed) infections.

Pneumonia status	Infection due to RSV	
	No	Yes
No/URTI	6644	0
Mild	635	68
Severe	258	61*
Very severe	29	5*
Total	7566	134

*these 2 categories were further classified as severe RSV LRTI

When RSV LRTI status was investigated in different age categories and at different cord levels, a significant difference (anova, *P=0.014*) was only noted between the number of episodes experienced by each age class. Cord levels had no effect. As noted from Table 8.3, in the under 6 and 12-24 month age classes the number of RSV LRTI episodes were similarly high, these numbers were observed to be higher than for the 6-11 month age class. As children

got older the incidence of RSV LRTI was seen to decrease. A trend of decline in severe RSV LRTI with age was noted, but this was not significant (anova, *P=0.127*) between age classes (Table 8.3).

Table 8.3. The number of RSV LRTI stratified by age class in months and cord levels

Cord levels	RSV LRTI				Severe RSV LRTI			
	< 6	6-11	12-24	24+	<6	6-11	12-24	24+
1	10	3	12	2	7	2	2	—
2	6	3	8	1	3	2	1	—
3	3	5	3	2	2	4	2	—
4	6	6	10	—	4	3	2	—
Total	25	17	33	5	16	11	7	—

8.5.6 Estimation of serological correlates of protection against RSV-associated LRTI

The mean of children who did not experience RSV-associated LRTI was compared to those who did using a Student's t-test with unequal variance. With regards to children who had RSV LRTI, approximate Ab titres (within 7 days of diagnosis) at onset of disease were used if no serum sample was available on day of diagnosis. It was found that the mean titres for those who did not experience RSV-associated disease was slightly greater (2.14 log AU) than for those who did experience disease (1.99 log AU). However, this did not attain statistical significance (*P= 0.191*). It did not matter if differences between children experiencing infection and those who did not were explored by individual age classes under 6 months as above, Ab titres for the non-infected population consistently remained higher in comparison although this did not reach statistical significance.

8.5.7 Incidence rates of IFAT and ELISA confirmed RSV-associated infections

The incidence rate of RSV infection over the whole follow up period at both the 2- and 4-fold ELISA-CSR levels as well as for IFAT-CSR were estimated to be 767, 494 and 403/1000 child years observation, respectively. Addtionally, the incidence of IFAT-CSR across all ages stratified by infection type *i.e.* primary, secondary and 2 infections or more was calculated to be 252, 110 and 41/1000 child years observation, respectively. As age increased, ELISA-CSR incident rates were observed to increase (Table 8.4). The relative hazard for infants 6-11, 12-23 and 24+ months of age compared to infants <6 months old was 3.3, 4.3 and 5.4 at the 2-fold seroconversion level, and 3.5, 5.0 and 6.7 at the 4-fold seroconversion level respectively (Table 8.5a & b). However, a general decline was noted in the incident rates for IFAT-CSR (Tables 8.4) until 12-23 months with an increase then being noted for children 24+ months of age. Children in age class 6-11, 12-23 and 24+ months having a reduced risk of 41, 56 and 26% respectively in comparison to those < 6 months old (Table 8.5c).

Table 8.4. Incident rates of ELISA-CSR and IFAT-CSR for different age groups

Age category (months)	Incidence rates/ 1000 children years observation (95% CI)		
	ELISA-CSR: 2-fold	ELISA-CSR: 4-fold	IFAT-CSR
<6	213 (156, 293)	121 (79, 183)	647 (540, 775)
6-11	699 (592, 825)	417 (336, 517)	383 (306, 479)
12-23	930 (842, 1026)	605 (535, 684)	287 (240, 343)
24+	1161 (980, 1376)	812 (663, 995)	480 (369, 625)

Table 8.5a. Exponential regression showing the relative harzard with age for ELISA-CSR at the 2-fold seroconversion level (each age group is compared to < 6 months group)

```
No. of subjects =          513                     Number of obs    =      3689
No. of failures =          706
Time at risk    =  920.7318243
                                                   LR chi2(3)       =    137.11
Log likelihood  =   -655.40553                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 6-11 months |   3.267788   .5921395     6.53   0.000      2.29093     4.66118
 12-23 months|   4.347199   .7296648     8.76   0.000     3.128512    6.040616
 24+ months  |   5.429922   .9887798     9.29   0.000     3.800066    7.758825
------------------------------------------------------------------------------
```

Table 8.5b. Exponential regression showing the relative harzard with age for ELISA-CSR at the 4-fold seroconversion level (each age group is compared to < 6 months group)

```
No. of subjects =          513                     Number of obs    =      3689
No. of failures =          455
Time at risk    =  920.7318243
                                                   LR chi2(3)       =    106.10
Log likelihood  =   -606.53134                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
6-11 months  |    3.45907   .8294756     5.18   0.000     2.161939    5.534459
12-23 months |   5.014039   1.113812     7.26   0.000     3.244165    7.749479
24+ months   |   6.730808   1.595744     8.04   0.000     4.229254      10.712
------------------------------------------------------------------------------
```

Table 8.5c. Exponential regression showing the relative harzard with age for IFAT-CSR (each age group is compared to < 6 months group)

```
No. of subjects =          513                     Number of obs    =      3667
No. of failures =          371
Time at risk    =  920.7318243
                                                   LR chi2(3)       =     40.36
Log likelihood  =   -810.66327                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
6-11 months  |    .591539   .0870034    -3.57   0.000      .443393    .7891834
12-23 months |   .4434099   .0572519    -6.30   0.000     .3442711    .5710976
24+ months   |   .7421428   .1211681    -1.83   0.068     .5389067    1.022025
------------------------------------------------------------------------------
```

A history of 1 or more previous infections was investigated and observed to affect both incidence rates of ELISA-CSR at both seroconversion levels, and IFAT-CSR across all age groups. The more infections experienced in the past, the higher the incident rate of RSV ELISA-CSR infections (Table 8.6).

Table 8.6. The effect of history of previous infection on general incidence rates of ELISA-CSR and IFAT-CSR

Previous # of infections	Incidence rates/ 1000 children years observation (95% CI)		
	ELISA-CSR: 2-fold	ELISA-CSR: 4-fold	IFAT-CSR
0	471 (427, 520)	329 (293, 371)	261 (229, 296)
1	3520 (3079, 4025)	2073 (1741, 2468)	4334 (3566, 5268)
2	3924 (3223, 4779)	2141 (1639, 2795)	5112 (3720, 7026)

On adjusting for age, 1 or 2 previous infections had a significant ($P<0.05$) effect on incident rates (Table 8.7a-c, non-shaded area). However, if age categories were taken into account, then adjusting for previous infections did not affect incident rates from one age category to the next. The rates were observed to be similar (Table 8.7a-c, shaded area).

Table 8.7a. Exponential regression showing the relative hazard after adjusting for indicated explanatory variables, for ELISA-CSR at the 2-fold seroconversion level

```
No. of subjects =          513                     Number of obs    =      3689
No. of failures =          706
Time at risk    =  920.7318243
                                                   LR chi2(5)       =    633.70
Log likelihood  =   -407.11028                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
1 past infecn |   6.659138   .5916123    21.34   0.000      5.59494    7.925754
2+ past infecn|   7.745882   .9440657    16.80   0.000     6.099956    9.835922
6-11 months   |    2.72735   .4964188     5.51   0.000     1.909015    3.896479
12-23 months  |   2.513188   .4338695     5.34   0.000     1.791744     3.52512
24+ months    |   2.227074     .42681     4.18   0.000     1.529696    3.242381
------------------------------------------------------------------------------
```

Note: Each infection (infecn) group is compared to children experiencing no infection; each age group is compared to < 6 months group

Table 8.7b. Exponential regression showing the relative hazard after adjusting for indicated explanatory variables for ELISA-CSR at the 4-fold seroconversion level

```
No. of subjects =          513                     Number of obs    =      3689
No. of failures =          455
Time at risk    =  920.7318243
                                                   LR chi2(5)       =    335.53
Log likelihood  =   -491.81786                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
1 past infecn |   5.250511   .5850247    14.88   0.000     4.220449    6.531975
2+ past infecn|   5.336568   .8471706    10.55   0.000     3.909627    7.284316
6-11 months   |   3.011761   .7241697     4.59   0.000     1.879972    4.824914
12-23 months  |   3.283962   .7433619     5.25   0.000     2.107265    5.117729
24+ months    |   3.367328   .8292306     4.93   0.000     2.078119    5.456327
------------------------------------------------------------------------------
```

Note: Each infection (infecn) group is compared to children experiencing no infection; each age group is compared to < 6 months group

Table 8.7c. Exponential regression showing the relative hazard after adjusting for indicated explanatory variables for IFAT-CSR

```
No. of subjects =          513                     Number of obs    =      3667
No. of failures =          371
Time at risk    =  920.7318243
                                                   LR chi2(5)       =    519.05
Log likelihood  =   -571.31574                     Prob > chi2      =    0.0000

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
1 past infecn |   17.62653   2.116942    23.89   0.000     13.92957    22.30467
2+ past infecn|   20.51741   3.650303    16.98   0.000     14.47716    29.07782
6-11 months   |   .4998068    .073795    -4.70   0.000      .374218    .6675436
12-23 months  |    .406901   .0526301    -6.95   0.000     .3157847    .5243079
24+ months    |   .4965793   .0825216    -4.21   0.000     .3585385    .6877672
------------------------------------------------------------------------------
```

Note: Each infection (infecn) group is compared to children experiencing no infection; each age group is compared to < 6 months group

Incidence rates of both ELISA- and IFAT-CSR appeared to be higher in the lower quartile of cord blood titres. Additionally, this rate appeared to increase even further in the upper quartile when IFAT-CSR was considered (Table 8.8).

Table 8.8. The effect cord levels on general incidence rates of ELISA-CSR and IFAT-CSR

Cord levels	Incidence rates/ 1000 children years observation (95% CI)		
	ELISA-CSR: 2-fold	ELISA-CSR: 4-fold	IFAT-CSR
1	873 (737, 1035)	619 (506, 757)	450 (355, 569)
2	659 (547, 795)	408 (321, 517)	366 (285, 470)
3	798 (671, 949)	468 (373, 587)	374 (291, 482)
4	782 (652, 938)	539 (433, 671)	526 (421, 657)

Note: cord levels 1-4 are lower, middle lower, middle upper and upper quartile ranges respectively.

It was noted that infants born within an epidemic had lower rates of infection (Table 8.9) although this did not attain statistical significance when comparing ELISA-CSR at both seroconversion levels ($P=0.36$ and $P=0.60$ at 2- and 4-fold seroconversion levels respectively). However, a significantly reduced risk of 21% ($P=0.022$) was noted for children born in an epidemic compared to those born outside the epidemic when IFAT-CSRs were taken into consideration (Table 8.10).

Table 8.9. The effect of being born in or out of an epidemic on general incidence rates of ELISA-CSR and IFAT-CSR

Born in epidemic	Incidence rates/ 1000 children years observation (95% CI)		
	ELISA-CSR: 2-fold	ELISA-CSR: 4-fold	IFAT-CSR
No	799 (714, 895)	509 (441, 586)	461 (397, 535)
Yes	745 (676, 821)	484 (429, 546)	363 (316, 417)

Table 8.10. Exponential regression showing the relative hazard of being born in an epidemic for IFAT-CSR (comparison to infants born outside an epidemic)

```
No. of subjects =          513                     Number of obs    =      3667
No. of failures =          371
Time at risk    =  920.7318243
                                                   LR chi2(1)       =      5.19
Log likelihood  =   -828.24424                     Prob > chi2      =    0.0227

------------------------------------------------------------------------------
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
Born in epid |   .7881394   .0820228    -2.29   0.022     .6427134    .9664709
------------------------------------------------------------------------------
```

8.6 Discussion

This chapter has described the immune profiles of individuals from birth through infancy and early childhood. There were patterns of Ab decay and acquisition of RSV-specific responses that groups of children appeared to experience. Cord blood levels were seen to vary, although generally, the decay of matAb appeared not to be altered by the presence of IFAT positively identified infections. In some instances, it appeared that the child did not experience any clinically confirmed infection although the assay was able to identify seroconversions (Figure 8.1a). These serology confirmed infection could have been due to subclinical infections. However, it should be borne in mind that asymptomatic infections which lead to the boosting of immunity can lead to an over-estimation of the duration of matAb protection against symptomatic disease (Wendelboe et al., 2005). On the other hand, other infants experienced 1 (Figure 8.1b) or > 1 (Figure 8.1c) clinically confirmed infections (IFAT-CSR). Post-infection, classical boosting of Ab titres was noted and either Ab titres were observed to remain elevated or waned. This may well account for the consistently high seroprevalence levels in this population. Alternatively, post-infection Ab titres were observed to decline either quickly or gradually.

Our study went on to further demonstrate a significant age-related difference in Ab responses to RSV infection during early life, *i.e.* the ability to develop an immune response to RSV was related to the age of the child; those less than 6-8 months of age did not consistently induce an immunological response (Figure 8.3) as similarly described previously (Chanock et al., 1961, McIntosh et al., 1978, Murphy et al., 1986a, Murphy et al., 1986b, Queiroz et al., 2002, Richardson et al., 1978, Welliver et al., 1980), and the proportion of children seroconverting was low, (~13%). Mean differences in Ab titres between paired acute and convalescent phase

samples thus were observed to be negative. Additionally, cord levels did not appear to influence the rate of seroconversion in this age group, but it seemed that the higher the matAb titres a child possessed *i.e.* cord blood level 4 (under 6 mo of age), the more suppressed their immune response (Figure 8.6b).

The apparent lack of a humoral immune response to RSV infection in children < 6 months, as previously described (Brandenburg et al., 1997) may therefore account for initial RSV-associated disease in the presence of matAbs. As well, this may account in part for the failure of the development of solid immunity allowing for subsequent re-infections by the virus (Murphy et al., 1986b). Other factors that include (i) masking by matAb or its interference with IgG-producing cells thus mediating temporary immunosuppression in infants; (ii) immaturity of the immune system; and (iii) the placentally acquired matAbs not possessing sufficient neutralizing activity against the circulating strains that caused the infection (Parrott et al., 1973, Queiroz et al., 2002) possibly due to strain specific deficiencies in matAb cover could also account for inefficient immune responses. This inadequate Ab response has implications for the design of an infant vaccine.

However, it is not possible to rule out entirely the possibility that the child's own immune response leads to protection against RSV-associated disease, but against the background of matAb remains indiscernible. A further complication is the definition of seroconversion in the presence of matAb. Simply, seroconversion conventionally refers to a change from negative (low pre-response serum) to positive (high post-response serum). However, as matAb are at adult levels and hence high, it is highly unlikely that an incompetent immune system will achieve titres above these levels. Therefore under 6 mo of age, it remains important to clearly

define the meaning of seroconversion, as this therefore has implications on calculating an effective protective threshold for vaccine efficacy. Is this the change in Ab titre above the matAb level at that particular time, or is this the change of Ab titres from the minimum baseline titres achieved following matAb decay?

Above 6 months of age, there appeared to be a dramatic increase in the proportion of children showing either a 2-or 4-fold IFAT-CSR. Additionally with each successive infection, mean convalescent titres were boosted further in comparison to the previous infection perhaps implying that each child attains his/her own protective threshold as convalescent titres seemingly approached adult levels (cord level titres). In some instances, re-infection with RSV in this older age class appeared to be associated with accelerated and more persistent development of RSV-specific IgG, which was most likely related to the disappearance of matAb from circulation. Cord levels had no effect on the immune responses above 11 months of life, although in the 6-11 month age bracket it appeared that the higher the cord titres a child initially had, seroconversion between paired samples occurred at a higher level than other cord levels (Figure 8.6b). This could be as a result of the child's immune system still being primed from the effects of matAbs and hence seroconversion occurs from this elevated level rather than the child's inherent baseline level.

A significant minority (21%) of children appeared not to seroconvert post-infection (Figure 8.3). This may be due to late sample collection, in other words by the time the convalescent serum was collected, acquired humoral immunity may have already declined. Previous studies on the kinetics of the development of Abs to structural proteins of Hantavirus following natural infection (Groen et al., 1992) demonstrated different levels of Abs to conserved

epitopes measured at different stages of the infection. The authors described the Abs as developing slowly and hence were either not detected or were detected at very low levels in acute and early convalescent sera, whilst in the convalescent phase, their concentration increased gradually, reaching the highest levels in late convalescent phase sera. Perhaps a similar mechanism exists for RSV and the convalescent samples collected were early phase. This may thus explain why it appeared that no seroconversion was noted for this subgroup of children.

The risk of re-infection with clinically identified RSV illness decreased with age, from an initial infection rate of 252 to 41/1000 child years observation. Previously, Murphy et al. (1986a, b) described an enhanced serum Ab response to RSV infection with age. Therefore, older infants and children experienced less re-infection when compared with infants less than 8 months of age. After adjusting for age, a history of 1 or more previous infections significantly increased ($P<0.05$) the relative hazard of a child experiencing further infection, whether IFAT-CSR or ELISA-CSR. This seems to imply that other host factors other than age at infection dictate a child's risk of infection. Our results are in contrast to Glezen et al. (1986) who previously observed a moderately lower risk for re-infection in children 24-35 months of age, although this did not attain statistical significance. Furthermore, very low cord titres resulted in an increased rate of RSV incidence. This therefore suggests that low matAb levels are not protective against RSV infection. It was also noted that very high titres of cord led to a further increase in incidence of clinically confirmed RSV-associated disease. It is hence plausible, that a protective threshold for matAbs against clinical RSV does exist, which thus allows for optimal protection but does not interfere with the child's subsequent immune response. Lastly, children born within an RSV season appeared to have a significantly

(*P=0.022*) reduced risk of infection by 21%. It thus appears that children born within an RSV season are born with higher matAbs as earlier inferred (section 7.6.7) and previously described by Le Saux and colleagues (2003).

A similar number of RSV LRTI episodes were noted in the under 6 and the 12-24 month age groups. It has previously been described that serious RSV-associated disease is most common in children 1-11 months old (Anderson et al., 1990, Kim et al., 1973, Parrott et al., 1973). Paradoxically, with respect to the lower age group, this is an age coincident with highest titres of maternally derived RSV Abs (Glezen, 1977, Glezen et al., 1981). Also, it is not entirely clear why the older age group in this cohort who are expected to have better developed immune systems, should experience similar levels of pneumonia as the under 6 month old children. A possible explanation for this is that these children lacked strain-specific Abs and were thus susceptible to serious RSV-associated disease. There exist several other factors that may contribute to the increased susceptibility to respiratory pathogens in the developing world and these include, indoor air pollution, overcrowding, low immunocompetence, poor nutrition and high nasopharyngeal carriage rates of potential bacterial pathogens (Forgie et al., 1991a, Forgie et al., 1991b).

A theoretical protective threshold against RSV LRTI of greater than 2.14 log AU is suggested. However, it should be noted that this level of Ab titres was only slightly higher than those established by the infected group. The numbers are too small to draw any definite conclusions. Moreover, a direct comparison to the threshold calculated by (Piedra et al., 2003a) who established a minimal protective threshold of titres $\geq$ 6.0 and $\geq$ 8.0 $\log_2$ (*i.e.* $\geq$1.81 and $\geq$2.41 $\log_{10}$ respectively) against RSV associated-hospitalization cannot be made as the authors

tested for neutralizing Abs to RSV A and B respectively. It thus still remains important to properly define the threshold level as this has implications for vaccine design.

The immune response remains multifaceted, involving both host and parasite factors, and their contribution to RSV-associated disease severity involves both magnitude and intensity of infection playing a crucial role, with this most likely being related to viral load. There exists evidence to suggest that the variable regions of RSV show progressive accumulation of amino acid changes which may be correlated with antigenic change (Cane & Pringle, 1995). Consequently, these antigens could induce protective immune responses that are poorly immunogenic *i.e.* weak inducers of immunity. It is also known that the various genotypes of RSV infecting children are highly variable, and hence immune responses to these strains may only be detectable if the target antigen accurately reflects the infecting virus (Cane et al., 1996; Scott et al., 2007). With regards to maternally acquired RSV-specific Abs, it has been suggested that their cross-reactive nature may be as a result of 2 different mechanisms or a combination of both. They may display strain-specific immunity as a result of mothers experiencing multiple infections involving different strains, or strain transcending immunity induced as a result of high levels of Abs against virally conserved epitopes also induced by repeat infections (Roca et al., 2002). Thus the authors argue that if the latter is the case, then mothers lacking Abs specific to the currently circulating virus strain leave their offspring vulnerable to infection. An appropriate vaccine should therefore limit infection in the mother reducing a major source of infection for the baby, as well, maternally transferred Abs across the placenta or by breast-feeding may confer protection to the infant. Nevertheless, a vaccine incorporating elements of both RSV group A and B would have to be considered.

With reference to the child's acquired immune response, it has long been thought that excessive expression of the latter plays a role in disease pathogenesis (Gardner et al., 1970). However, recently it was shown that severe RSV LRTI is characterized by inadequate adaptive responses, robust viral replication and apoptotic crisis (Welliver et al., 2007). Above 6 months of age many children were noted to seroconvert in the absence of clinical symptoms, clearly reflecting the presence of protective immunity in such children. On the other hand, other children became ill with RSV from time to time, implying that some strains are better tolerated than others, as resistance to severe RSV-associated disease increases with age (Brandt et al., 1973, Glezen et al., 1986, Kim et al., 1973). This seems to suggest that infants develop resistance to severe RSV-associated disease at a much slower rate than children, and likewise, children at slower rates compared to adults. Could it be then that the constitutive difference related to immune responses between children and adults and by extension between infants and children, rather than the differential exposure to a sufficient number of protective antigenic variants, as recently debated for the acqusition of protective immunity to malaria (Hviid, 1998) account for the differences noted between infants and children, and children and adults? No doubt, the relative importance of the various types of immune responses which include responses to variable and conserved targets (Roca et al., 2003; Cane et al., 1996) in the acquisition and maintenance of protective immunity play a part in the overall response to infecting strains and is host dependent. More studies, particularly longitudinal studies, are needed to therefore resolve the ambiguities of the immune response to RSV infection.

Future experiments should consider the use of more sensitive biological assays to discern between rising titres following infection in the presence of matAbs. Assays that can test for RSV specific glycoproteins, F & G, especially IgA F and G should be sought. This can be

achieved in one of two ways; firstly by using the neutralizing assay. This would give a clearer picture of the development of immune response with age. However, these assays have been said to measure only a sub-set of Abs which cannot be assumed to include all biologically relevant Abs (McGill et al., 2004). An alternative assay is the membrane fluorescence assay. This allows for the use of clinical isolates, which replicate to low titres in cell culture, to be used as the antigen (McGill et al., 2004, Scott et al., 1976) as well as allowing for higher seroconversion rates to be measured. Additionally, as the assay measures Abs to unfixed, cell surface proteins thus recognizing Abs to conformation dependent epitopes (Routledge et al., 1988) this will give a clearer picture of the sub-set of biologically relevant Abs involved in the clearance of RSV infection, giving the best assessment of immunity currently available in the child (McGill et al., 2004). Additionally, other immunoglobulins should be tested for, both in sera and NW samples collected during the entire follow-up period.

To summarize, the components of the immune response to RSV, resulting in protective immunity or pathogenesis of the infection still remains to be fully elucidated and have hence hindered vaccine development (Anderson & Heilma, 1995, Kimpen, 1996). However, Abs to both surface proteins, G and F are thought to be necessary for efficient neutralization of RSV infection (Hendry et al., 1988), a potential vaccine must be able to prime protective Abs to the intended virus in mothers who no doubt will have already experienced a previous infection with a related viral strain. In this manner, maternal:foetal transfer would be efficient and following RSV infection, the child would most likely attain or realize near or complete protection to severe RSV-associated disease, as their immune response is primed optimally. An optimal immune response would stimulate both IgG and IgA Abs working at maximal synergy against both F and G proteins of RSV. An immune response to either protein would

consequently lead to partial protection allowing for re-infection. The interaction to immunodominant epitopes however, depends on the recognition of its amino acid sequence in conjunction with that of overlapping or adjacent putative receptor sites. These must thus remain unmasked and active *i.e.* not have undergone any mutation, for complete protection. Crucial to all this, is a minimal protective threshold of pre-existing antibody, whether matAb during primary infection, or the child's own repertoire during re-infection, must be realized to induce complete protection. Host responses remain important and should also be taken into consideration during vaccine design to tease out whether it is the need for several successive infections, or the constitutive difference related to immune responses in the host, that leads to more complete protection from RSV-associated disease.

CHAPTER NINE
Overall Discussion

RSV is the most common cause of childhood viral LRTI in both the developed and developing world. It was earlier noted that serum Ab had protective effects against RSV infection (Glezen et al., 1981, Groothuis et al., 1995, The IMpact-RSV study group, 1998,), and there was a correlation with decreasing levels of IgG RSV Ab in maternal blood and increased risk of infection in the off-spring (Roca et al., 2002). Despite this understanding, a precise correlate of immunity has not been defined as yet. In order to implement the usage of a RSV vaccine, knowledge of the prevalence of RSV infection in all age groups is important, as this not only will give a clearer picture of the time of infection, but also allows for the estimation of the appropriate age for vaccination.

9.1 Findings and supporting evidence

The main objectives of this study were to examine the roles of passive and acquired Ab to RSV infection in infants and young children in Kenya, a developing country. The study was carried out from 2002-2005. In summary, the main findings from this research are:

1. Approximately 97% of infants were born with RSV-specific matAbs, which was indicative of efficient matAb transfer during the last trimester of gestation, although these levels were variable.
2. Although it appeared that seasonal changes had no bearing on matAb levels and levels were similar in children born in or out of an RSV epidemic period, children born within an RSV season appeared to have a significantly (*P=0.022*) reduced risk of IFAT-CSR of 21%. Additionally, RSV-specific matAb titres were significantly lower

for infants who went on to experience infection (IFAT-CSR or ELISA-CSR) within the first 6 months of life in comparison to those who did not.

3. The rates of RSV-specific matAb decay for both the infected and non-infected population groups were shown to be the same; however RSV-specific cord levels significantly influenced these rates.

4. RSV matAb $T_{1/2}$ was calculated to be around 79 days with a mean duration of 111 days, whilst loss of seropositivity over the first 6 months of life occurred in a delayed manner, with 50% of children still being seropositive by 6 months.

5. By 12 and 24 months, 54% and 85% of infants had experienced their first and second RSV infection respectively as identified by IFAT.

6. The majority of children under the age of 6 months of age, despite having clinically confirmed infections (IFAT-CSR), did not show any seroconversion. Above 6 months of age, some children were observed to experience boosting of Ab titres (ELISA-CSR) in the absence of virologically confirmed infections. Yet others experience one or more virologically confirmed infection (IFAT-CSR). Ab responses showed considerable variability. With each successive infection, convalescent titres were boosted towards adult levels.

7. RSV A comprised 98% of the circulating strain in epidemic 1, RSV A and B were similarly distributed in epidemic 2, whilst in both epidemics 3 and 4 RSV A comprised 100% and 98%, respectively of circulating subgroup. The same strain was seen to cause both RSV-associated URTI and LRTIs.

8. The risk of re-infection with clinically identified RSV illness decreased with age, from an initial infection rate of 252 to 41/1000 child years observation. A history of 1 or

more previous infections, having adjusted for age, resulted in a significant increase in incidence of RSV infection, identified by both ELISA- and IFAT-CSR.

Thus, data from this study lend further support for the ubiquitous nature of RSV in Kilifi, Kenya as similarly reported in other parts of the world, representing a major health burden.

A simple regression model to calculate the rate of decline in RSV matAb was used. These data were slightly lower than what has been previously been reported in the literature (Cox et al., 1998, Hacimustafaoglu et al., 2004, Ward et al., 1983), except for Brandenburg et al. (1997) who reported a much lower rate of 26 days.

9.2 Generalisability of study findings

RSV is one of the important WHO recognized diseases causing a major burden and targeted for vaccine control (www.who.int/vaccine_research/diseases/portfolio/en/index.html). As thus, there remains an urgent need for a better understanding of the clinical impact of RSV in developing nations (Robertson et al., 2004, Wright & Cutts, 2000). The study demonstrated that at a population level, matAb declined rapidly over the first 6 months of age as expected and subsequently, a gradual increase in Ab titres was seen with increasing age and infection as previously noted (Chanock et al., 1961, McIntosh et al., 1978, Murphy et al., 1986a, Murphy et al., 1986b, Queiroz et al., 2002, Richardson et al., 1978, Welliver et al., 1980). At the individual level, it was noted that each child experienced its own characteristic immune response (Figure 8.1). However, the establishment of a protective threshold against RSV-associated disease and the rate of loss of seroprevalence following both the loss of matAb and acquired immunity, remain important parameters for vaccine design. This community-based

longitudinal study has given important insight into the Ab dynamics in a developing country and from the data a correlate for immunity can be inferred, as well as the appropriate age for vaccination. It seems likely therefore that vaccination in early childhood, which would thus delay infection to the 2nd year or later when the child's immune system is more mature, may be sufficient enough for control of severe RSV-associated disease in early childhood. However, it is highly doubtful that Abs induced by vaccination would be more durable than those that arise following natural infection as was previously reported for measles, whereby measles vaccines elicited lower Ab titres than that observed after natural disease (Black, 1989, Jenks et al., 1988).

9.3 Limitations of the study

(i) Frequency of sampling: it is possible that due to the quick decay of Ab titres, a small proportion of infections may have been missed or were not adequately captured due to the 3 monthly sampling frame. As well, the 1 month interval between paired samples did not allow for optimal capture of Ab dynamics. Shorter sampling frequencies may thus resolve this, allowing for better interpretation of RSV-specific Ab kinetics especially in the first year of life and especially following infection (IAFT-CSR and ELISA-CSR), as post-infection, not all resulting immune responses persisted. Some responses were observed to sharply decline to a minimum even within a period of 2 weeks. A shorter sampling frame however, may still prove difficult due to ethical issues.

(ii) Measures of immunity: the measurement of ELISA binding titres using a crude extract is an indirect measurement of functional Abs. It would have been more suitable to measure NtAb titres to get a direct measurement of functional Ab following

infection and in the presence of matAbs. Furthermore, the use of A2 rather than the currently circulating strain in the assay procedure could have led to an under estimation of seroconversion due to poor cross-reactivity (Roca et al., 2003).

(iii) Problems in identifying the infecting virus: we showed that many infections during the study period were identified by serology but were not detected by clinical surveillance (ELISA-CSR). However, repeated attempts to measure RSV by PCR in nasal samples collected in the interverning period between 2 consecutive 3 monthly sera that were seen to show seroconversions (ELISA-CSR) and might have accounted for these changes in Ab titres, proved futile. The use of more sensitive assays, such as real time PCR or the establishment of collected NW in culture as a means of viral amplification could improve sensitivity. The cell culture laboratory and real time PCR analysis are now established in Kilifi and this would now be possible.

9.3.1 Limitations in our understanding of the problem

There still exist many deficiencies in our understanding of the duration of RSV-specific immunity as well as the variable response of infants after natural infection as also noted by previous studies (Cox et al., 1998, Fisher et al., 1997, Kasel et al., 1987, Murphy et al., 1986a, Murphy et al., 1986b, Wagner et al., 1989). This may be in part be due to the fact that there is no clear seological marker for protective immunity against RSV. Most importantly however, these data as with previous studies (Murphy et al., 1986a, Queiroz et al., 2002) suggest that age plays an important role affecting the infant's ability to develop RSV-specific immune responses, it appears that at least by 3 years postinfection, immunity against severe RSV-associated disease is established, but, immunity against re-infection is not attained. Studies controlling for the levels of circulating RSV in the population are important, as asymptomatic

infections can boost the level of immunity and can thus lead to an overestimation of the duration of protection against symptomatic disease (Wendelboe et al., 2005). Other factors contributing to the difficulty in measuring immune responses as well as the limited comparability between studies include the use of different assays, the ultilization of different case definitions, surveillance methods and reporting systems.

Further studies to distinguish between the interplay of waning immunity and boosting of RSV acquired immunity post-infection, together with the transmission dynamics in the population, will aid determination of the optimal age and frequency of immunizations and hence their role in RSV control, as it may then be possible to improve the control of the disease through an optimal vaccination strategy which takes into accont both waning immunity and earlier susceptibility of infants (Rouderfer et al., 1994) to be implemented.

9.4 Future work

The restricted humoral responses in young children following RSV infection should be taken into consideration during vaccine development. Furthermore, HIV has an important impact on the immune response and RSV-associated disease, resulting in immune suppression and would therefore cause exacerbated disease in immunocompromised individuals, as well as causing prolonged shedding (Madhi et al., 2001). Therefore future studies should aim to collect data on HIV status. Moreover, the measurement of these humoral responses should not be restricted to only IgG, but other Ig classes as a means of distinguishing between matAb responses and the child's own response, especially under 6 month of age, in addition to distinguishing between primary and other immune responses.

Due to the quick decline in matAb as well as the loss of acquired Ab, it is necessary to visit cohort children at shorter intervals in order to get better estimates on the rate of loss of RSV-specific antibody. Furthermore, it is pertinent to establish the titres of NtAb to circulating strains using such assays as MFAT and future studies should take this into consideration. Additionally, regular sampling even in the non-epidemic periods should be considered, as repeat exposure to virus probably has an important role in boosting Ab titres. A constant supply of susceptible individuals would predict transmission of infection, and hence the observed clinical epidemics would be generated by strain variation (Cox et al., 1998). This would give an indirect indication of circulating strain, allowing for one to establish the role of cross-protectiveness. This in turn would inform on the protective threshold with regards to circulating strain, rather than the prototype, that should be attained to give protection against RSV disease.

Finally, in light of the fact that efficient transfer of matAbs from mother-to-child occurs, and the quick decay of the latter as well as of acquired Ab thus allowing for re-infections, it could be suggested that to minimize severe RSV disease, maternal vaccination should be considered; as well, children would then have to be vaccinated at a later age, perhaps repeatedly. These alternatives to vaccine strategies should be further explored. The optimal age for vaccination will depend on the definition of the minimal protective/interfering titre and this depends on vaccine strain and dose (Whittle et al., 1988).

In conclusion, it appears that matAbs do offer some protection although a small proportion of infected infants are seen to develop LRTI and may require hospitalization. A number of isolates of RSV were seen to co-circulate in the community, with the same strain causing both

URTIs and LRTIs, humoral immune responses and Ab seroconversion between acute and convalescent infant sera were demonstrable for all age groups tested.

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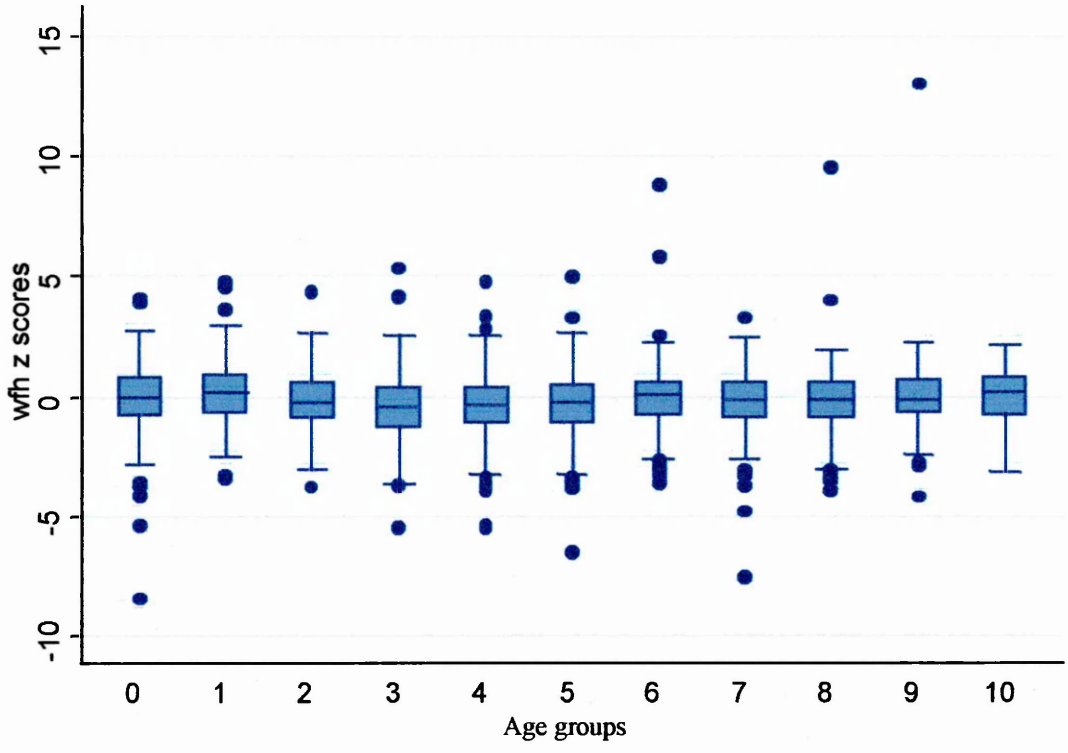
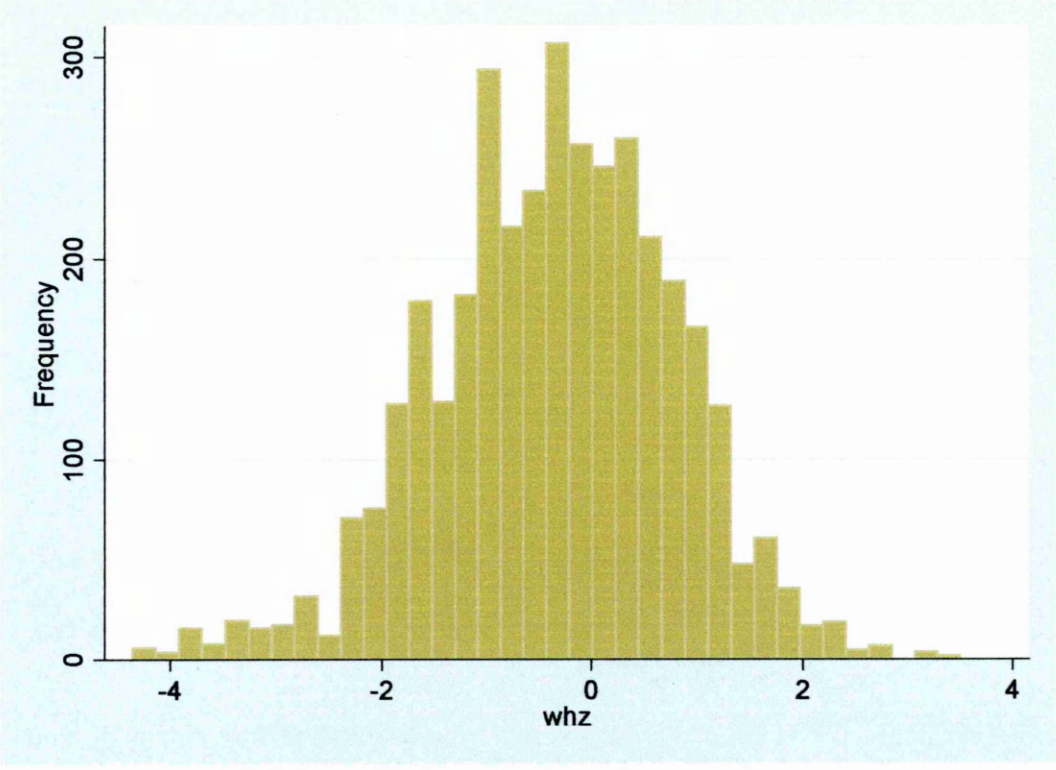
APPENDIX I
WHO child growth standards



Note: wfh: weight-for height z scores; Age groups 0: 0-89; 1: 90-179; 2: 180-269; 3: 270-359; 4: 360-449; 5: 450-539; 6: 540- 629; 7: 630-719; 8: 720-809; 9: 810- 899; 10: ≥ 900 days respectively

Histogram of weight-for-height for children in cohort



APPENDIX II
RSV Study: home visit form

Date today ____ / ____ / ____

Next appointment ____ / ____ / ____

KBC-No.	NAME1	NAME2	NAME3	SEX	FLWK	TEMP	RSV+2wk	BDWK	KEYSGNWK	BDOBS	KEYSGNOB	NWREQ	BRATE	REFER	AWAYW
3245	JUMA	CHE	ALI	M	N	36.41	N	Y	C+DC	N	F+C	Y 10:00am	60/min	Y 30/=	2wks

*(compiled by Emelda Okiro, MJ Ngama, DJames Nokes)

Key signs: Difficulty in breathing (DB), Nasal Discharge/Congestion (DC), Cough (C), Fever (F)
Breathing rate: fast breathing=50 breaths/minute or 40 breaths/minute in children >11 months (*i.e.* 1 year and above)

APPENDIX III
RSV Study: sick child clinic visit

QUESTIONNAIRE

FIELD WOKER

complete page 1 + record Oxygen Sat/Pulse/Weight/Axillary temp on page 2

KBC # [ ][ ][ ][ ][ ]

RSV # [ ][ ][ ]

Child Name [ ] [ ] [ ]

Date of Birth [ ][ ]/[ ][ ]/[ ] [ ] dd/mm/yy

Date today [ ][ ]/[ ][ ]/[ ] [ ] dd/mm/yy

Visit type [ ] active (referral by FW) [ ] passive (self-referral) - Tick one box

---

IF ACTIVE REFERRAL *(ie referred by FW from home) - Refer to clinician to complete only pages 2-3*

IF PASSIVE REFERRAL (ie mother self referred) - FW complete the below

1. Ask mother - has child had a fever in the last 7 days? [ ] y/n
2. Has the child a temperature >37.49C (see page 2) [ ] y/n

If either 1 or 2 is 'yes' then do blood slide.

3. Blood slide taken [ ] y/n
4. Blood slide result [ ] +/-
5. Ask mother - has the child had difficulty in breathing in last 7 days? [ ] y/n
6. Ask mother - which of the key symptoms in the last 7 days? [ ]
7. Observe the child to have difficulty breathing today? [ ] y/n
8. Observe which key symptoms today ? [ ]
9. Is a nasal washing required? [ ] y/n
10. NW time [ ][ ]:[ ][ ] hh:mm
11. NW result [ ] +/-
12. Breathing rate (cycles / min) [ ][ ]
13. Does child have fast breathing for age [ ] y/n
14. Refund single (whole shillings) [ ][ ] ksh

Key symptoms: cough c, fever f, nasal discharge/congestion dc

Now refer child to clinician .....next page

KBC# [ ][ ][ ][ ][ ] RSV# [ ][ ][ ]

To be completed by CLINICIAN

HISTORY

How long has the child been sick? .....[ ][ ] DAYS

What is/are the main problems?(Please answer Y/N)

FEVER.....[ ]

COUGH.....[ ]

RUNNING NOSE/NASAL DISCHARGE/NASAL CONGESTION.....[ ]

DIFFICULTY IN BREATHING.....[ ]

OTHERS(specify below).....[ ]

PHYSICAL EXAMINATION

OXYGEN SATURATION	[ ][ ][ ]%	FW to complete
AXILLARY TEMPERATURE	[ ][ ].[ ][ ]C	
WEIGHT	[ ][ ].[ ][ ]kg	
HEART RATE	[ ][ ][ ]/min	

COUGH HEARD	[ ] Y/N
HOARSENESS	[ ] Y/N
SORETHROAT	[ ] Y/N
RHINITIS	[ ] Y/N
PHARYNGITIS	[ ] Y/N
CYANOSIS	[ ] Y/N
EAR INFECTION	[ ] Y/N
CONJUCTIVITIS	[ ] Y/N
PROSTRATED	[ ] Y/N
RESPIRATORY RATE	[ ][ ]/min
NASAL FLARING	[ ] Y/N
INDRAWING	[ ] Y/N
CRACKLES	[ ] Y/N
WHEEZES	[ ] Y/N
STRIDOR	[ ] Y/N

APPENDIX IV

Information and consent form for birth cohort child.

STUDY OF A MAJOR CAUSE OF PNEUMONIA IN KILIFI DISTRICT

Information and consent form for birth cohort child.

PURPOSE OF STUDY

Chest infections are one of the biggest health problems for children in Kilifi District. We know the cause and the right treatment for many of these, but in some cases this is not yet known. We are investigating an infection caused by a germ (virus) that is known to be a major cause of chest infections throughout the world. The virus is called Respiratory Syncytial Virus or RSV. For infants who are infected in their first year of life the symptoms can be severe enough for the parents to feel a need to take the child to hospital. This condition is distressing both to the infant and to the family, although is rarely life threatening. There are no drugs in routine use for reducing the symptoms, and currently no vaccine to prevent infection. However, much work is being carried out to develop a vaccine to prevent infection.

The purpose of this study is to identify how important is RSV in Kilifi District, and to find out how the virus is transmitted and maintained within the population. Results of both would be of great value in developing and eventually implementing a vaccine against this disease.

WHO IS ORGANISING THE STUDY

The study is organised by the Kenya Medical Research Institute (KEMRI) CGMRC, Kilifi District Hospital (KDH) in collaboration with groups from outside Kenya (Universities in England) who have specific knowledge of the disease under study.

YOUR INVOLVEMENT

You are already enrolled in a Birth Cohort Study of Malaria and Pneumonia (pneumococcal) disease run by KEMRI CGMRC. We are asking you to involve your newly born child in another study of pneumonia in which we will monitor the child for infections of the nose and lungs that may be due to RSV. The method of monitoring is EITHER through you bringing your child to the hospital when he / she has symptoms of a cold or a chest infection, OR through weekly visits during the outbreaks of these problems (which occur each year) of our survey staff to your house to check on the child's health. If you decide to enroll we will record information on your pregnancy, details of your child (weight, length), and household information (location, number of siblings, household details).

We will monitor your child until the end of year 2004 which is a period of three years [insert 2 years as appropriate].

If the child has signs of nose or chest infection that might be due to RSV we will collect a nasal specimen (fluid from the nose) in which we will check for the virus. If our tests confirm that the child is infected with RSV we will request a small sample of blood (less than half a teaspoon) and saliva soon after and again after 3-4 weeks by which to see how the child's immune defenses are reacting.

Additionally, every 3 months we will collect a sample of saliva. You have already agreed for blood samples to be collected from your child every three months as part of the malaria and pneumococcal pneumonia study. For this RSV study, we would like to use a small amount of

these blood samples and {where appropriate} we would also like you to continue to bring your child in for 3 monthly blood sampling until the end of the study period. The samples are to look for evidence of the infection.

{Where appropriate} We will also ask for a swab from the nose and throat each week in the first year during the main season of RSV, to assist in identifying the disease.

HOW DO WE COLLECT SAMPLES

Nasal specimens will be collected by washing out the nasal passage with some harmless fluid. The fluid is squirted into one nostril using a syringe [demonstrate device] and collected as it returns through the same or the other nostril. This procedure, conducted by trained staff, causes no harm to the infant, and whilst being a little uncomfortable, should clear the nasal passage making breathing more easy.

We collect saliva using a small clean sponge on the end of a plastic stick [show the device]. The sponge is brushed around the teeth and gums for half a minute - like using a toothbrush. This is painless, and without risk. Our staff will show how it is done.

Blood collection is by trained personnel. We will collect a sample of 2ml as much as will fit in this tube [demonstrate a 2ml tube].

{Where appropriate} Nasal and throat swabs are collected using cotton buds on the end of a wire support. This brushed against the back of the throat (throat swab [show device]) or inserted into the nose and brushed against the wall of the inner nose (nasal swab [show device]).

RISKS AND BENEFITS OF THE STUDY

All of the tests described above (taking blood or collecting fluid from the nose or mouth) are commonly used tests in clinics in many countries. They carry no danger for your child. Some of the procedures may be slightly uncomfortable, we try to ensure that this is not a problem by using highly trained staff to perform the tests.

Your child will benefit from having coughs, chest infections and fever investigated more quickly than would usually be the case because they are being seen more often. If your child is unwell at any time during the study, s/he can attend the research clinic at KDH and we will provide, without charge, examination and routine investigations (such as malaria tests) and medications (such a treatment for malaria, chest, skin or ear infections). Throughout the study period if our field worker asks you to attend the clinic with the child because s/he is unwell, or you bring the child to the clinic because you yourself have identified signs of nose or lung infection in the child (signs that we will explain), we will refund reasonable costs of your return travel fare. Because our funds are strictly limited we can only offer these benefits to the child involved in the study.

The other main benefit of research studies is in helping to improve understanding of disease, which may lead to better ways of preventing and treating these diseases. This benefits children and families in Kilifi District as a whole, as well as in other parts of Kenya.

WHAT HAPPENS TO THESE SAMPLES AND THE INFORMATION COLLECTED

The samples are sent to the laboratory at Kilifi Hospital where they will be tested to identify if your child is infected with RSV, or has had the infection in the recent past. To protect the privacy of your child, we will keep the records of this study under a code number rather than by name. We will keep the records in locked files and only study staff will be allowed to look

at them. The name of your child or other facts that might point to him/her will not appear when we present this study or publish its results.

We would also like to store any blood/saliva/nasal washings that are left over after we do your tests. We plan to use these samples for studies we may do in the future. The samples will be labeled with a number, not a name, and if further information is required, e.g. to identify that this sample comes from your child, this information will only be provided to the researchers if the new study has been scrutinised and approved by the National Ethical Committee in KEMRI. You can decline to let us store your child's blood and saliva for use in other research projects and still be in this study.

MORE INFORMATION

Please feel free to ask any questions about the study. You are free to join in the study or not. You may also decide to withdraw your child from the study at any time, for any reason. If, after discussing this with us, you decide to allow your child to join the study we would like you to sign this form.

We have given you a copy of this consent form. When you sign below, it shows that you agree to join the study. If there is any part of this form which you do not understand be sure to ask questions about it. Do not sign until you have full answers to all your questions. When you are ready to be part of the study, please tick one of the two boxes and print and sign your name on the lines below.

Consent form for infant cohort study.

STUDY OF A MAJOR CAUSE OF PNEUMONIA IN KILIFI DISTRICT

To Mother

- Have you read, or had read to you, the invitation letter?
- Have you had an opportunity to ask questions and discuss this study?
- Have you received satisfactory answers to those questions?
- Have you received enough information about the study?
- Do you understand that you are free to withdraw your child from the study
  - at any time
  - without having to give a reason why
  - without affecting your child's access to medical provision

I wish my child [name] _____to take part in the study

☐ **I also give consent for my child's blood/saliva/nasal samples to be stored at KEMRI under the conditions above**

☐ **I do not give consent for my child's blood to be stored at KEMRI for future research**

Print Name: _____

Signature _____ Date _____

Household number/registration _____

Witness

I observed the process of consent. The parent/guardian of the prospective participant read this form, was given the chance to ask questions, appeared to accept the answers, and signed to enroll his/her child in the study.

Name: _____

Signature _____ Date _____

The study co-ordinator is Dr James Nokes. If you have any questions about the ethics of this study you can contact Dr Charles Mbogo, at KEMRI/Wellcome Trust, Kilifi District Hospital or telephone Kilifi 22535.

APPENDIX V
Sources of Reagents, Equipment and Consumables

Reagents

Supplier	Reagent
Alta Bioscience, Birmingham, UK	1st round MPX PCR primer mix
	2nd round MPX PCR primer mix
	N gene primer mix
Ambion (Europe) Ltd, Huntingdon, Cambridgeshire	RNase Zap®
Amersham Biosciences, Buckinghamshire, UK	pd(N)$_6$ Random hexamer 5'-phospate, sodium salt
BDH (Merck Ltd), Leicester, UK	Hydrogen peroxide
	Tween-20
Birmingham University, Division of Immunity & Infection, Medical School,UK	RSV A2 strain (ATCC VR-1540)
Chemicon International, Inc, USA	Light Diagnostics™ Respiratory Syncytial Virus direct immunofluorescence assay (DFA)
Dako, Cambridgeshire, UK	Goat anti-mouse IgG-HRP gamma-chain spec (2 mL)
	Rabbit anti-human IgG HRP gamma-chain spec (2 mL)
European Cell Culture Collection, Porton Down, UK	HEp-2 cells (ECACC 86030501/CCL-23)
	RSV A2 strain (ATCC VR-1540)
Fisher Scientific, Leicestershire, UK	Absolute ethanol
	Acetone
	Sulphuric acid
Gibco-BRL Life Technologies Ltd, Paisley, UK	Agarose (electrophoresis grade)
	MEM Non essential amino acids (100X), liquid without L-Glutamine
	Minimal Essential Media (MEM) with Earles salts+ L-Glutamine
	D-PBS powder (10 L)
	PBS 0.01 pH 7.2 (100 tablets)
	penicillin/streptomycin
	Trypsin-EDTA (1X)
Invitrogen Ltd. Paisley, UK	Agarose

Supplier	Reagent
New England Biolabs (UK), Herts, UK	Hind III (10,000 Units)
	Pst I (10,000 Units)
	Bgl II (10,000 Units)
	Hae III (3,000 Units)
	Rsa I (1,000 Units)
Qiagen Ltd. West Sussex, UK	QIAamp Viral RNA Mini kit
	Omniscript Reverse Transcriptase kit
	Taq PCR Master Mix kit
Sigma-Aldrich Co Ltd, Dorset, UK	Amphotericin B
	Ethidium bromide
	Fetal bovine serum, certified (heat inactivated) 500ml
	HEPES buffer solution (1M)
	Nonidet P40 substitute
	O-phenylendiamine dihydrochloride tables (30 mg)
Tescos Supermarket, UK	Marvel Dried skimmed milk
Warwick University, UK	Hep2 cells

Equipment and Consumables

Supplier	Equipment/Consumable
Alpha Laboratories Ltd, Hampshire, UK	Apex 2.0 mL microtubes
	Apex screw cap white
BD Biosciences, Cambridge UK	BD Falcon ™ 15 mL high-clarity polypropylene conical centrifuge tube
Gilson Medical Electronics, France	Gilson pipetman: P10, P100, P200, P1000
Dynex Technologies, Ashford, UK	Immulon–2 HB flat bottom 96 well microtitre plates
Eppendorf, Cambridge, UK	Eppendorf Centrifuge 5415D
	Multichannel pipettor
Jencons, Bedfordshire, UK	Cryovials 1.2 mL
Fisher Scientific, Leicestershire, UK	Nalgene Mr. Frosty
	Tissue culture flask: 125cm^2 + 0.2um filtered caps, 75cm^2 + 0.2 um filtered caps, 25cm^2 angle neck+ 0.2um filtered caps
	Cell Scraper blade length 320 mm blade width 17.5 mm
Phillip-Harris Scientific, UK	Glass balls 3.7-4.1mm undrilled (500g)
Sarstedt, Leicester, UK	Aeroseal filter tip autoclavabale: 10 µL, 100 µL, 200 µL, 1000 µL
	Microcentrifuge tube natural polypropylene with safety cap: 0.5 mL, 1.5 mL
	Centrifuge tubes: 15 mL, 50 mL
	Pipette tips: blue (1000 µL), yellow (200 µL)
	Serological sterile pipette: 1 mL, 5 mL, 10 mL, 25 mL
	Transparent ELISA plate sealers
Sigma-Aldrich Co Ltd, Dorset, UK	IKA mini MS1 mixer

APPENDIX VI
Growth stages non-infected and infected cultured HEp-2 cells

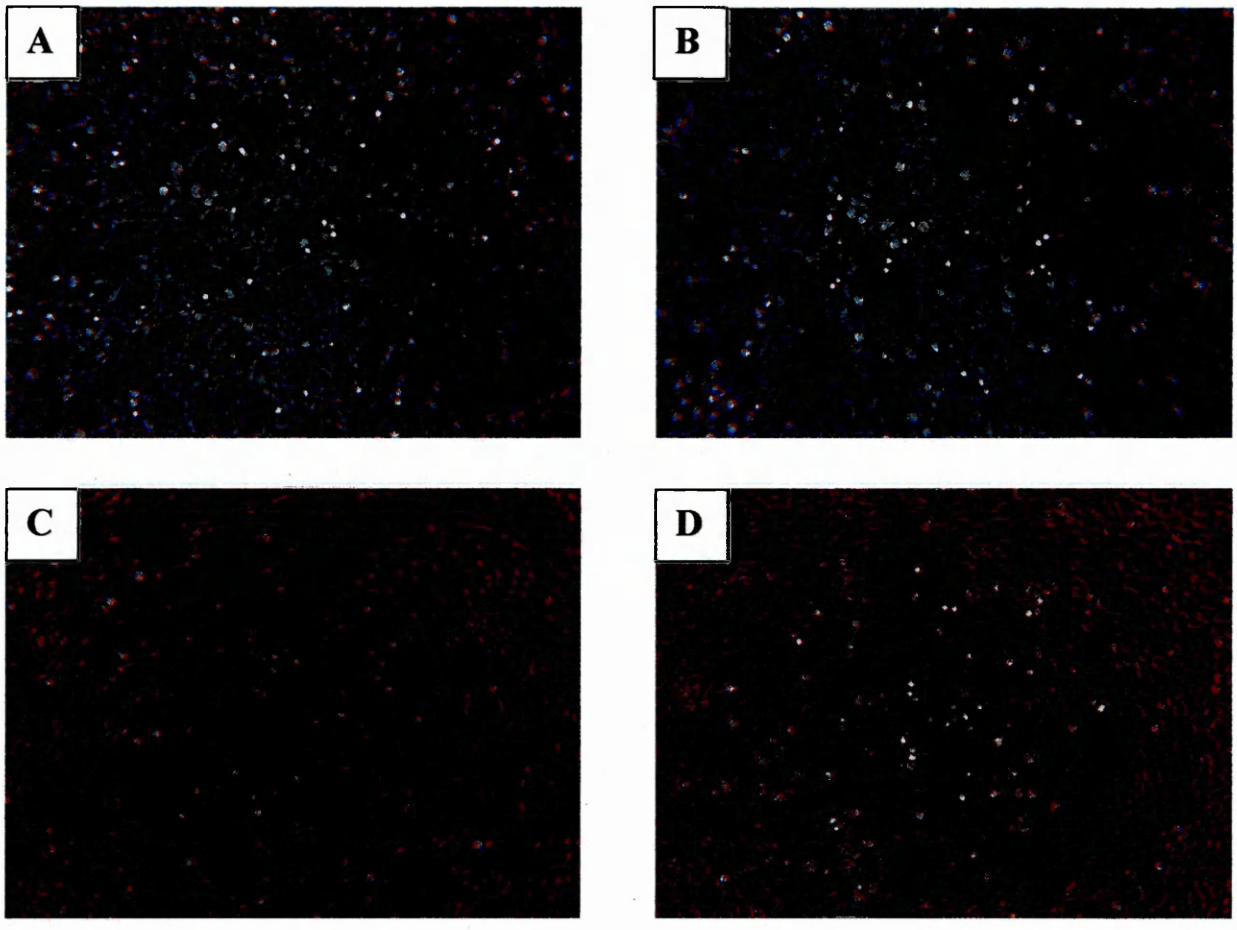


Photo: Panels A-B, day 3 and panels C-D, day 4 HEp-2 cells post-inoculation with RSV A2. Panels A and C and panels B and D are infected cells and mock-infected (control) cells respectively. (Magnification: X100 objective)

APPENDIX VII

Sequences of primers used and amplicon sizes

(Cane & Pringle, 1991, Stockton et al., 1998)

Primer	Gene Position	Sequence 5' to 3'	Amplicon size (bp)
N Gene Primer Mix			
N1	N	GGAACAAGTTGTTGAGGTTTATGAATATGC	
N2	N	CTTCTGCTGTCAAGTCTAGTACACTGTAGT	
1st Round MPX Primer Mix			
AH1 A	HA	CAGATGCAGACACAATATGT	1, 015
AH1 F11	HA	AAACCGGCAATGGCTCCAAA	
RSV AB F	N	GTCTTACAGCCGTGATTAGG	838
RSV AB R	P	GGGCTTTCTTTGGTTACTTC	
BHA D11	HA	TGTTTTCACCCATATTGGGC	900
BHA A	HA	GTGACTGGTGTGATACCACT	
AH3 A	HA	CAGATTGAAGTGACTAATGC	883
AH3 D11	HA	GTTTCTCTGGTACATTCCGC	
2nd Round MPX Primer Mix			
AH1 B	HA	ATAGGCTACCATGCGAACAA	944
AH1 E11	HA	CTTAGTCCTGTAACCATCCT	
AH3 B	HA	AGCAAAGCTTTCAGCAACTG	591
AH3 C11	HA	GCTTCCATTTGGAGTGATGC	
BHA B	HA	CATTTTGCAAATCTCAAAGG	767
BHA C11	HA	TGGAGGCAATCTGCTTCACC	
RSV A F	N	GATGTTACGGTGGGGAGTCT	334
RSV A R	N	GTACACTGTAGTTAATCACA	
RSV B F	N	AATGCTAAGATGGGGAGTTC	183
RSV B R	N	GAAATTGAGTTAATGACAGC	

APPENDIX VIII
Modified Lysate preparation/ELISA procedure

(Note: Modifications to assays indicated in bold)

Materials

Nonidet-P40 (NP40)
PBS
Cell scraper
15 mL falcon tube
Vibra-Cell™ ultra-sonicator (Sonics & Materials, Inc)
Nunc-Immuno™ MaxiSorp™

1. When extensive CPE (50-75%) is noted in infected HEp-2cells, scrape cells into medium, and place in a centrifuge tube, similarly for control flasks.
2. Spin at 352 X g for 10 min and then decant off supernatant (CAUTION: Infectious)
3. Resuspend pellet in 10 mL PBS, spin at 352 X g for 10 min.
4. Decant off supernatant (CAUTION: Infectious).
5. Resuspend in 10 mL sterile water + 0.5% NP40 and vortex very hard.
6. Spin at 352 X g for 10 min.
7. **Place 10 mL of supernatant into 15 mL falcon tube previously cut into half and then sonicate at 70% amplitude, 3X 1 min cycles with 1 sec pulse and 1 sec pause (to allow for maximal virus liberation from cells).**
8. **Pool respective lysates and then vortex hard.**
9. Aliquot supernatant in 2ml quantities and store at –70°C until further use.

Plate Preparation

1. Dilution of lysate must first be determined. Dilute above lysate *e.g.* 1:2, 4, 8, 16, 32, 64 (both infected and uninfected) in **PBS (coating buffer).** Add **25** µl to each well (always do wells in triplicate) of 96-well **MaxiSorp™** plates.
2. **Allow lysate to adsord to wells over night at 37°C in a rotating incubator (to ensure optimal binding of proteins and hence reduce non-specific binding).**
3. **Flick off coating buffer and proceed with ELISA assay below.**

ELISA Assay

1. Block plates with 200 µL/well with 5% Marvel in **PBS** and then seal plates and incubate for 1 hr at 37°C.
2. Flick off block and bang plate on paper towel.
3. Meanwhile dilute sera and control (adult serum) in block (*i.e.* 5% Marvel in **PBS**) 1:100
4. Also carry out serial dilution of standard (pooled adult sera) starting 1:50 to 1:1600
5. Add 100 µL/well of serum, control in every plate and one set of standard per run, all in duplicate, and re-seal plates and incubate for 1.5 hr at 37°C.
6. Wash plates using plate washer 6 times with 200 µLl PBS-T.
7. Dilute the secondary antibody, goat anti-human HRP conjugated 1:1000 in block and add 100 µL to each well.
8. Incubate at 37°C for 1 hour and wash as above.
9. Develop with 100 µL O-phenylenediamine per well (30 mg tablet in 30 mL PBS, add 30 µL fresh H_2O_2) for 10 min. Prepare just before use. (Care: carcinogenic).
10. Add 50 µL 2.5 M H_2SO_4 per well to quench the reaction.
11. Read at 492 nm.

APPENDIX IX
Distribution of nasal wash sample results and type of samples collected

(a) Table 1. NW results from entire follow up period

NW results	Frequency	Percent
Negative	7,905	93.1
Equivocal	110	1.3
No result	21	0.2
Positive	457	5.4
TOTAL	8,493	100.0

(b) Table 2. Frequency of type of samples collected from infants during entire follow up period

Sample type	**Frequency**	**Percent**
Cord	361	10
3 month	2,765	75
Acute	295	8
Convalescent	270	7
Total	3,691	100

(c) Table 3. The number of sera samples per child available under 6 months of life used to carry out regression analysis/antibody response model

No of samples	No of children	Percent
1	503	41.54
2	422	34.85
3	207	17.09
4	58	4.79
5	18	1.49
6	3	0.25
Total	1211	100

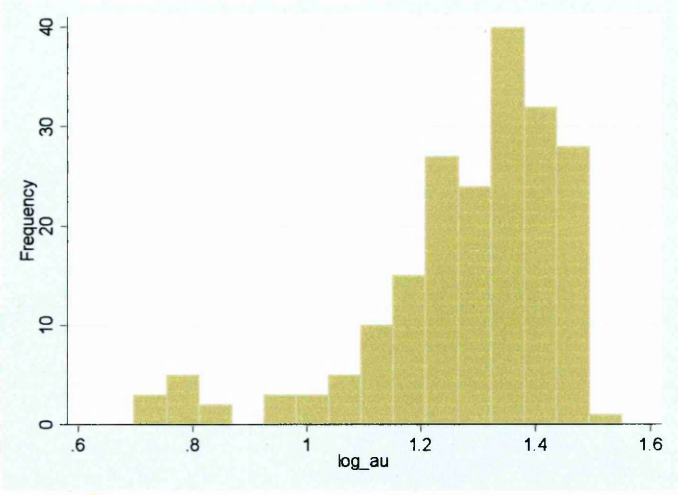
APPENDIX X
Stem-and- leaf plot for r (Studentized residuals)

```
-4* | 41
-3. | 7665
-3* | 4432211
-2. | 87
-2* | 443322111100000
-1. | 99999888887776666666666555555555555555555
-1* | 44444444444444433333333333333332222222222222222211111111111111 ... (90)
-0. | 99999999999999999999998888888888888888888888888888888888888 ... (197)
-0* | 44444444444444444444444444444444444444444444444444444444444444 ... (248)
 0* | 00000000000000000000000000000000000000000000000000000000000000 ... (309)
 0. | 55555555555555555555555555555555555555555555555555555555555555 ... (281)
 1* | 00000000000000000000000000000000000000000000011111111111111111 ... (202)
 1. | 55555555555555555555555555556666666666666666666666677777777777 ... (123)
 2* | 00000000000000001111111111111122222222222222222233333333333 ... (92)
 2. | 55555555555555555555556666666666666666666667777777777777777 ... (114)
 3* | 00000000000000000000011111111111111111112222222222222222222 ... (90)
 3. | 55555555555555555566666666666666666777777777777777888888888888 ... (83)
 4* | 00000000000000000000000001111111111111111111112222222222222 ... (106)
 4. | 55555555555555555556666666666666666667777777777777777888 ... (102)
 5* | 00000000000000011111111111111111122222222223333333333333344444 ... (72)
 5. | 55555555555555566666666666666667777777777777777788888888888888 ... (102)
 6* | 00000000000000000000011111111111111111111112222222222222222 ... (98)
 6. | 55555555555555555555666666666666666666666667777777777777777777 ... (90)
 7* | 00000000000000000001111111111112222222222222222222222333 ... (100)
 7. | 55555555555555555566666666666666666666667777777777777778888 ... (99)
 8* | 00000000000000000000011111111111111111111112222222223333333 ... (80)
 8. | 55555555555555555555556666666666666666667777777777777777777 ... (94)
 9* | 00000000000011111111111122222222222333333333333333344444444444
 9. | 55555555555566666666666667777777777777777888888888889999999999
10* | 00000000000011111112222222222222222223333333333333333333333 ... (83)
10. | 55555555555555566666666666677777777777777888888899999
11* | 00000000000000111111111111122222222333333333333444444444444444
11. | 55555666666666777777777888888888999999999999999
12* | 00000000000111111111122222222233333333444444444
12. | 555555555556666666777777778888888999999
13* | 000000001111111111222222333333333444444444
13. | 5555555555556666666667777788888899999999
14* | 00000000011111122233333333344444444444
14. | 555555556666666677777778888888999999999999
15* | 000000111111111111222222222333333333444444444
15. | 55555555566666677777888899999999
16* | 00000111111111133333444444
16. | 555555556666666777778888899
17* | 0001111111222333444
17. | 5555566667779
18* | 0002234444
18. | 557788
19* | 012344
19. | 577
20* | 1224
20. | 57788
21* |
21. | 8
22* | 04
22. | 6
23* | 0
23. |
24* |
24. |
25* |
25. |
26* |
26. |
27* |
27. |
28* |
28. |
29* | 2
```

Note: Outlier indicted by green ellipse

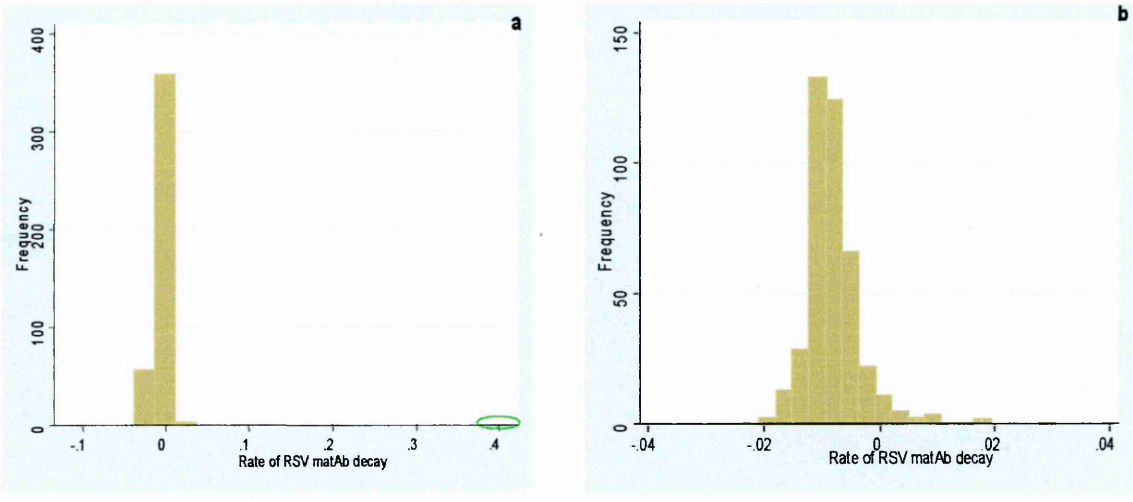
APPENDIX XI

(a) Histogram of seronegative population under 6mo of life



Mean for seronegative population is 1.224 logAU, this was used in the regression analysis (Table 7.4) where all infants below the seropositive cut-off value had their antibody titres adjusted to the mean of the seropositive population

(b) A histogram of gradients of infants under 6 mo of life, (a) showing outliers encircled with green elliptical circle and (b) with outliers omitted.



APPENDIX XII
Student t-tests

(a) Gradients

(i) Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples, at 2-fold seroconversion level (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     316   -.0090059    .0002029    .0036069   -.0094051   -.0086067
       1 |     106   -.0069163    .0014075    .0144915   -.0097072   -.0041254
---------+--------------------------------------------------------------------
combined |     422    -.008481    .0003862    .0079331   -.0092401   -.0077219
---------+--------------------------------------------------------------------
    diff |           -.0020896    .0014221               -.004908    .0007288
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.4694
Ho: diff = 0                     Satterthwaite's degrees of freedom =  109.393

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0723         Pr(|T| > |t|) = 0.1446          Pr(T > t) = 0.9277
```

(ii) Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples, at 4-fold seroconversion level (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     322   -.0088983    .0002078    .0037282    -.009307   -.0084895
       1 |     100   -.0071374    .0014838    .0148381   -.0100816   -.0041933
---------+--------------------------------------------------------------------
combined |     422    -.008481    .0003862    .0079331   -.0092401   -.0077219
---------+--------------------------------------------------------------------
    diff |           -.0017608    .0014983               -.0047324    .0012107
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.1752
Ho: diff = 0                     Satterthwaite's degrees of freedom =  102.908

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1213         Pr(|T| > |t|) = 0.2426          Pr(T > t) = 0.8787
```

(iii) Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples, at 2-fold seroconversion level of kids born **outside** an epidemic (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     119   -.0084241    .0002889    .0031511   -.0089961   -.0078521
       1 |      52   -.0072467    .0006688    .0048231   -.0085895    -.005904
---------+--------------------------------------------------------------------
combined |     171   -.0080661    .0002878    .0037638   -.0086342   -.0074979
---------+--------------------------------------------------------------------
    diff |           -.0011774    .0007286               -.0026301    .0002754
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.6160
Ho: diff = 0                     Satterthwaite's degrees of freedom =  70.7368

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0553         Pr(|T| > |t|) = 0.1105          Pr(T > t) = 0.9447
```

(iv)Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples, at 2-fold seroconversion level of kids born **inside** an epidemic (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     197   -.0093573    .0002722    .0038211   -.0098942   -.0088204
       1 |      54   -.0065981    .0026993    .0198356   -.0120122    -.001184
---------+--------------------------------------------------------------------
combined |     251   -.0087637    .0006189    .0098056   -.0099827   -.0075448
---------+--------------------------------------------------------------------
    diff |           -.0027592     .002713               -.0081982    .0026797
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.0171
Ho: diff = 0                     Satterthwaite's degrees of freedom =  54.0822

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1568         Pr(|T| > |t|) = 0.3137          Pr(T > t) = 0.8432
```

Neither were differences in rate of decay between the 2 population groups observed when stratified by epidemic at 4-fold seroconversion level

(v) Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples at 2-fold seroconversion level of kids with a **birth weight level > 2.5 kg** (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     237   -.0090565    .0002296    .0035349   -.0095088   -.0086041
       1 |      86   -.0080838    .0016109    .0149386   -.0112867    -.004881
---------+--------------------------------------------------------------------
combined |     323   -.0087975    .0004597    .0082615   -.0097019   -.0078931
---------+--------------------------------------------------------------------
    diff |           -.0009727    .0016272               -.0042061    .0022607
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.5978
Ho: diff = 0                     Satterthwaite's degrees of freedom =  88.4761

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2758         Pr(|T| > |t|) = 0.5515          Pr(T > t) = 0.7242
```

(vi)Two-sample t-test with unequal variances of **gradients** of infected & non-infected samples at 4-fold seroconversion level of kids with a **birth weight level > 2.5 kg** (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     242   -.0089596    .0002337    .0036353     -.00942   -.0084993
       1 |      81   -.0083131    .0017018    .0153163   -.0116998   -.0049264
---------+--------------------------------------------------------------------
combined |     323   -.0087975    .0004597    .0082615   -.0097019   -.0078931
---------+--------------------------------------------------------------------
    diff |           -.0006466    .0017178               -.0040631      .00277
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.3764
Ho: diff = 0                     Satterthwaite's degrees of freedom =  83.0356

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.3538         Pr(|T| > |t|) = 0.7076          Pr(T > t) = 0.6462
```

(b) Cord blood titres

(i)Two-sample t-test with unequal variances of **cord blood titres** of infected & non-infected samples at 2-fold seroconversion level of kids born **outside** an epidemic (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      89    3.038385    .0491793    .4639563    2.940652    3.136119
       1 |      30    2.962086    .1058686    .5798664    2.745561    3.178612
---------+--------------------------------------------------------------------
combined |     119     3.01915    .0453069    .4942397     2.92943     3.10887
---------+--------------------------------------------------------------------
    diff |            .076299    .1167338               -.1592431    .3118412
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.6536
Ho: diff = 0                     Satterthwaite's degrees of freedom =  42.2183

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7415         Pr(|T| > |t|) = 0.5169          Pr(T > t) = 0.2585
```

(ii) Two-sample t-test with unequal variances of **cord blood titres** of infected & non-infected samples at 4-fold seroconversion level of kids born **outside** an epidemic (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      92    3.017189    .0515181    .4941441    2.914855    3.119524
       1 |      27    3.025833    .0969801    .5039234    2.826488    3.225179
---------+--------------------------------------------------------------------
combined |     119     3.01915    .0453069    .4942397     2.92943     3.10887
---------+--------------------------------------------------------------------
    diff |           -.008644    .1098146               -.2302913    .2130033
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.0787
Ho: diff = 0                     Satterthwaite's degrees of freedom =  41.7939

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.4688         Pr(|T| > |t|) = 0.9376          Pr(T > t) = 0.5312
```

(iii) Two-sample t-test with unequal variances of **cord blood titres** of infected & non-infected samples at 2-fold seroconversion level of kids with a **birth weight level < 2.5 kg** (Group 0 and 1 denote non-infected and infected populations respectively)

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      79    2.946175    .0636349     .565599    2.819488    3.072863
       1 |      15     2.65532    .2478656    .9599793    2.123702    3.186939
---------+--------------------------------------------------------------------
combined |      94    2.899762    .0667244    .6469172    2.767261    3.032264
---------+--------------------------------------------------------------------
    diff |            .290855    .2559038               -.2519311    .8336412
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   1.1366
Ho: diff = 0                     Satterthwaite's degrees of freedom =  15.8939

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8637         Pr(|T| > |t|) = 0.2726          Pr(T > t) = 0.1363
```

(iv) Two-sample t-test with unequal variances of **cord blood titres** of infected & non-infected samples at 4-fold seroconversion level of kids with a **birth weight level < 2.5 kg** (Group 0 and 1 denote non-infected and infected populations respectively

```
------------------------------------------------------------------------------
   Group |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |       80    2.938939    .0632497    .5657229    2.813043    3.064834
       1 |       14    2.675898    .2653313    .9927787    2.102684    3.249111
---------+--------------------------------------------------------------------
combined |       94    2.899762    .0667244    .6469172    2.767261    3.032264
---------+--------------------------------------------------------------------
    diff |            .2630409    .2727659               -.3200542     .846136
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.9643
Ho: diff = 0                     Satterthwaite's degrees of freedom =  14.5117

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8247         Pr(|T| > |t|) = 0.3507          Pr(T > t) = 0.1753
```

(v) Two-sample t-test with unequal variances of **cord blood titres** of infected & non-infected samples at 4-fold seroconversion level of kids with a **birth weight level > 2.5 kg** (Group 0 and 1 denote non-infected and infected populations respectively

```
------------------------------------------------------------------------------
   Group |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      213    3.032248    .0341252    .4980407     2.96498    3.099516
       1 |       54    2.892726     .087026     .639508    2.718174    3.067279
---------+--------------------------------------------------------------------
combined |      267     3.00403    .0325179    .5313462    2.940005    3.068055
---------+--------------------------------------------------------------------
    diff |            .1395213    .0934776               -.0469074      .32595
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   1.4926
Ho: diff = 0                     Satterthwaite's degrees of freedom =  70.1374

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9300         Pr(|T| > |t|) = 0.1400          Pr(T > t) = 0.0700
```

APPENDIX XIII
Multiple regression models

In all subsequent analysis, bw_level implies birth weight level; e~2fold_level and e~4fold_level, infection at the 2-and 4-fold levels respectively; born_in_epi, birth in or out of epidemic period; _Iquart_le~2 to le~4, cord levels 1-4 (*i.e.* lower, middle lower, middle upper and upper quartile ranges respectively) and _Iparity_l~2 to l~3, parity levels 2-3 (ie 4^{th}-6^{th} birth and > 6 births respectively)

(a) Gradients

(i) Controlling for birth weight level & infection at 2-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     209
-------------+------------------------------           F(  2,   206) =    0.71
       Model |  .000046452     2  .000023226           Prob > F      =  0.4909
    Residual |  .006701236   206   .00003253           R-squared     =  0.0069
-------------+------------------------------           Adj R-squared = -0.0028
       Total |  .006747688   208  .000032441           Root MSE      =   .0057

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    bw_level |  -.0007008   .0009701    -0.72   0.471    -.0026133    .0012118
e~2fold_le~1 |   .0008743   .0008153     1.07   0.285    -.0007332    .0024818
       _cons |  -.0084243   .0008632    -9.76   0.000    -.0101262   -.0067224
------------------------------------------------------------------------------
```

No difference at 4-fold seroconversion level either

(ii) Controlling for birth in or out of epidemic period & infection at 2-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     209
-------------+------------------------------           F(  2,   206) =    0.92
       Model |  .000059629     2  .000029814           Prob > F      =  0.4008
    Residual |  .006688059   206  .000032466           R-squared     =  0.0088
-------------+------------------------------           Adj R-squared = -0.0008
       Total |  .006747688   208  .000032441           Root MSE      =   .0057

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 born_in_epi |  -.0007745   .0008037    -0.96   0.336     -.002359     .00081
e~2fold_le~1 |   .0006355   .0008102     0.78   0.434     -.000962    .0022329
       _cons |  -.0084415   .0007189   -11.74   0.000    -.0098588   -.0070242
------------------------------------------------------------------------------
```

No difference at 4-fold seroconversion level either

(iii) Controlling for parity levels & infection at 2-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     201
-------------+------------------------------           F(  3,   197) =    0.62
       Model |  .000061847     3  .000020616           Prob > F      =  0.6042
    Residual |  .006572738   197  .000033364           R-squared     =  0.0093
-------------+------------------------------           Adj R-squared = -0.0058
       Total |  .006634585   200  .000033173           Root MSE      =  .00578

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iparity_1~2 |   .0006905   .0009913     0.70   0.487    -.0012645    .0026455
_Iparity_1~3 |   .0012502   .0015288     0.82   0.414    -.0017647    .0042652
e~2fold_le~l |   .0007423   .0008304     0.89   0.372    -.0008953    .0023799
       _cons |  -.0092237   .0006007   -15.36   0.000    -.0104083   -.0080392
------------------------------------------------------------------------------
```

No difference at 4-fold seroconversion level either

(iv) Controlling for cord blood titres & infection at 2-fold seroconversion level

```
cord levels     _Iquart_lev_1-4     (naturally coded; Iquart_lev_1 omitted)

      Source |       SS       df       MS              Number of obs =     181
-------------+------------------------------           F(  4,   176) =   34.92
       Model |  .000831496     4  .000207874           Prob > F      =  0.0000
    Residual |  .001047813   176  5.9535e-06           R-squared     =  0.4424
-------------+------------------------------           Adj R-squared =  0.4298
       Total |  .001879309   180  .000010441           Root MSE      =  .00244

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iquart_le~2 |  -.0030211   .0005178    -5.83   0.000     -.004043   -.0019992
_Iquart_le~3 |  -.0043159   .0005289    -8.16   0.000    -.0053596   -.0032721
_Iquart_le~4 |   -.005967   .0005346   -11.16   0.000    -.0070221   -.0049118
e~2fold_le~l |   .0006289   .0003857     1.63   0.105    -.0001323    .0013901
       _cons |  -.0054697   .0004192   -13.05   0.000    -.0062969   -.0046424
------------------------------------------------------------------------------
```

(v) Controlling for cord blood titres & infection at 4-fold seroconversion level

```
cord levels     _Iquart_lev_1-4     (naturally coded; _Iquart_lev_1 omitted)

      Source |       SS       df       MS              Number of obs =     181
-------------+------------------------------           F(  4,   176) =   34.32
       Model |  .000823533     4  .000205883           Prob > F      =  0.0000
    Residual |  .001055776   176  5.9987e-06           R-squared     =  0.4382
-------------+------------------------------           Adj R-squared =  0.4254
       Total |  .001879309   180  .000010441           Root MSE      =  .00245

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iquart_le~2 |  -.0030468   .0005196    -5.86   0.000    -.0040724   -.0020213
_Iquart_le~3 |  -.0043251   .0005315    -8.14   0.000    -.0053741   -.0032761
_Iquart_le~4 |  -.0059972    .000536   -11.19   0.000    -.0070551   -.0049393
e~4fold_le~l |   .0004512   .0003941     1.15   0.254    -.0003265    .0012289
       _cons |  -.0053829   .0004181   -12.87   0.000     -.006208   -.0045577
------------------------------------------------------------------------------
```

(vi) Controlling for all potential risk factors & infection at 2-fold seroconversion level

```
parity levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)
cord levels     _Iquart_lev_1-4     (naturally coded; _Iquart_lev_1 omitted)

      Source |       SS       df       MS              Number of obs =     173
-------------+------------------------------           F(  8,   164) =   15.89
       Model |  .000770581     8  .000096323           Prob > F      =  0.0000
    Residual |  .000994422   164  6.0635e-06           R-squared     =  0.4366
-------------+------------------------------           Adj R-squared =  0.4091
       Total |  .001765003   172  .000010262           Root MSE      =  .00246

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 born_in_epi |  -.0003358   .0003962    -0.85   0.398    -.0011182    .0004466
    bw_level |   .0001587   .0004831     0.33   0.743    -.0007951    .0011125
_Iparity_l~2 |   .0003959   .0004533     0.87   0.384    -.0004991    .0012908
_Iparity_l~3 |    .00003   .0007078     0.04   0.966    -.0013675    .0014276
_Iquart_le~2 |  -.0028759   .0005426    -5.30   0.000    -.0039473   -.0018045
_Iquart_le~3 |  -.0043093   .0005621    -7.67   0.000    -.0054192   -.0031993
_Iquart_le~4 |  -.0058579   .0005557   -10.54   0.000    -.0069551   -.0047608
e~2fold_le~1 |   .0005562     .00041     1.36   0.177    -.0002534    .0013657
       _cons |  -.0055597   .0006569    -8.46   0.000    -.0068569   -.0042626
------------------------------------------------------------------------------
```

(vii) Controlling for all potential risk factors & infection at 4-fold seroconversion level

```
parity_levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)
cord levels     _Iquart_lev_1-4     (naturally coded; _Iquart_lev_1 omitted)

      Source |       SS       df       MS              Number of obs =     173
-------------+------------------------------           F(  8,   164) =   15.63
       Model |  .000763591     8  .000095449           Prob > F      =  0.0000
    Residual |  .001001412   164  6.1062e-06           R-squared     =  0.4326
-------------+------------------------------           Adj R-squared =  0.4050
       Total |  .001765003   172  .000010262           Root MSE      =  .00247

------------------------------------------------------------------------------
 _b_age_days |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 born_in_epi |  -.0003438   .0003976    -0.86   0.388    -.0011288    .0004412
    bw_level |   .0002143   .0004831     0.44   0.658    -.0007396    .0011682
_Iparity_l~2 |   .0003823   .0004549     0.84   0.402    -.0005158    .0012804
_Iparity_l~3 |   .0000434   .0007119     0.06   0.951    -.0013622    .0014491
_Iquart_le~2 |  -.0029115   .0005443    -5.35   0.000    -.0039861   -.0018368
_Iquart_le~3 |  -.0043424    .000565    -7.68   0.000    -.0054581   -.0032267
_Iquart_le~4 |  -.0058945   .0005567   -10.59   0.000    -.0069936   -.0047953
e~4fold_le~1 |   .0003458   .0004186     0.83   0.410    -.0004808    .0011724
       _cons |  -.0054901   .0006579    -8.34   0.000    -.0067892    -.004191
------------------------------------------------------------------------------
```

(b) Cord blood titres

(i) Controlling for birth weight level & infection at 2-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     361
-------------+------------------------------           F(  2,   358) =    5.70
       Model |  3.54282361     2  1.77141181           Prob > F      =  0.0037
    Residual |  111.233148   358  .310707118           R-squared     =  0.0309
-------------+------------------------------           Adj R-squared =  0.0255
       Total |  114.775972   360  .318822144           Root MSE      =  .55741

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    bw_level |   .1176612   .0670007     1.76   0.080    -.0141032    .2494255
e~2fold_le~l |  -.2181391   .0728353    -2.99   0.003    -.3613779   -.0749003
       _cons |   2.934572   .0586556    50.03   0.000     2.819219    3.049925
------------------------------------------------------------------------------
```

(ii) Controlling for birth weight level & infection at 4-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     361
-------------+------------------------------           F(  2,   358) =    3.62
       Model |  2.27641504     2  1.13820752           Prob > F      =  0.0277
    Residual |  112.499557   358  .314244572           R-squared     =  0.0198
-------------+------------------------------           Adj R-squared =  0.0144
       Total |  114.775972   360  .318822144           Root MSE      =  .56058

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    bw_level |   .1131323   .0673514     1.68   0.094    -.0193218    .2455864
e~4fold_le~l |   -.166283   .0755924    -2.20   0.028     -.314944   -.0176221
       _cons |   2.924528   .0589048    49.65   0.000     2.808685    3.040371
------------------------------------------------------------------------------
```

(iii) Controlling for birth in or out of epidemic period & infection at 2-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     361
-------------+------------------------------           F(  2,   358) =    4.92
       Model |  3.07057252     2  1.53528626           Prob > F      =  0.0078
    Residual |  111.705399   358  .312026255           R-squared     =  0.0268
-------------+------------------------------           Adj R-squared =  0.0213
       Total |  114.775972   360  .318822144           Root MSE      =  .55859

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 born_in_epi |  -.0783123    .062752    -1.25   0.213    -.2017211    .0450965
e~2fold_le~l |   -.217065   .0730721    -2.97   0.003    -.3607695   -.0733604
       _cons |   3.073873    .054419    56.49   0.000     2.966852    3.180894
------------------------------------------------------------------------------
```

(iv) Controlling for birth in or out of epidemic period & infection at 4-fold seroconversion level

```
      Source |       SS       df       MS              Number of obs =     361
-------------+------------------------------           F(  2,   358) =    2.86
       Model |  1.80733401     2  .903667005            Prob > F      =  0.0584
    Residual |  112.968638   358  .315554854            R-squared     =  0.0157
-------------+------------------------------           Adj R-squared =  0.0102
       Total |  114.775972   360  .318822144            Root MSE      =  .56174

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 born_in_epi |  -.0725221   .0630448    -1.15   0.251    -.1965067    .0514625
e~4fold_le~1 |  -.1647097   .0757952    -2.17   0.030    -.3137695   -.0156499
       _cons |   3.056522   .0542906    56.30   0.000     2.949753    3.16329
------------------------------------------------------------------------------
```

(v) Controlling for parity levels & infection at 2-fold seroconversion level

```
parity levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)

      Source |       SS       df       MS              Number of obs =     348
-------------+------------------------------           F(  3,   344) =    3.73
       Model |  3.36668764     3  1.12222921            Prob > F      =  0.0116
    Residual |  103.505417   344  .300887841            R-squared     =  0.0315
-------------+------------------------------           Adj R-squared =  0.0231
       Total |  106.872105   347  .307988775            Root MSE      =  .54853

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iparity_l~2 |   .0634102   .0695672     0.91   0.363    -.0734204    .2002407
_Iparity_l~3 |   .1146711    .109699     1.05   0.297    -.1010941    .3304362
e~2fold_le~1 |  -.2246853   .0735484    -3.05   0.002    -.3693465   -.0800241
       _cons |   2.991718   .0390545    76.60   0.000     2.914902    3.068534
------------------------------------------------------------------------------
```

(vi) Controlling for parity levels & infection at 4-fold seroconversion level

```
parity levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)

      Source |       SS       df       MS              Number of obs =     348
-------------+------------------------------           F(  3,   344) =    2.36
       Model |  2.15829552     3   .71943184           Prob > F      =  0.0710
    Residual |  104.713809   344  .304400609            R-squared     =  0.0202
-------------+------------------------------           Adj R-squared =  0.0117
       Total |  106.872105   347  .307988775            Root MSE      =  .55173

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iparity_l~2 |   .0708385   .0698812     1.01   0.311    -.0666097    .2082868
_Iparity_l~3 |   .1173114   .1104075     1.06   0.289    -.0998474    .3344702
e~4fold_le~1 |  -.1753541   .0764933    -2.29   0.022    -.3258075   -.0249008
       _cons |   2.976745   .0387356    76.85   0.000     2.900557    3.052933
------------------------------------------------------------------------------
```

(vii) Controlling for all parameters & infection at 2-fold seroconversion level

```
parity levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)

      Source |       SS       df       MS              Number of obs =     348
-------------+------------------------------           F(  5,   342) =    2.86
       Model |   4.2845187     5   .85690374            Prob > F      =  0.0153
    Residual |  102.587586   342  .299963703            R-squared     =  0.0401
-------------+------------------------------           Adj R-squared =  0.0261
       Total |  106.872105   347  .307988775            Root MSE      =  .54769

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    bw_level |   .1060265   .0685473     1.55   0.123    -.0288008    .2408538
 born_in_epi |  -.0368811   .0635888    -0.58   0.562    -.1619553    .0881932
_Iparity_l~2 |   .0653385   .0695399     0.94   0.348    -.0714412    .2021182
_Iparity_l~3 |   .1073177   .1097712     0.98   0.329    -.1085941    .3232295
e~2fold_le~l |  -.2366209    .073806    -3.21   0.001    -.3817918     -.09145
       _cons |   2.939824    .082255    35.74   0.000     2.778034    3.101613
------------------------------------------------------------------------------
```

(viii) Controlling for all parameters & infection at 4-fold seroconversion level

```
parity levels   _Iparity_le_1-3     (naturally coded; _Iparity_le_1 omitted)

      Source |       SS       df       MS              Number of obs =     348
-------------+------------------------------           F(  5,   342) =    1.96
       Model |  2.98023236     5  .596046471            Prob > F      =  0.0837
    Residual |  103.891873   342  .303777405            R-squared     =  0.0279
-------------+------------------------------           Adj R-squared =  0.0137
       Total |  106.872105   347  .307988775            Root MSE      =  .55116

------------------------------------------------------------------------------
      log_au |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    bw_level |   .1020746   .0689668     1.48   0.140    -.0335778    .2377271
 born_in_epi |  -.0313095   .0639353    -0.49   0.625    -.1570654    .0944465
_Iparity_l~2 |    .073159   .0698888     1.05   0.296     -.064307     .210625
_Iparity_l~3 |   .1109427   .1105297     1.00   0.316    -.1064608    .3283463
e~4fold_le~l |  -.1856068   .0767014    -2.42   0.016    -.3364727   -.0347409
       _cons |   2.923366   .0824405    35.46   0.000     2.761212    3.085521
------------------------------------------------------------------------------
```